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16. ABSTRACT

Baculoviruses appear to be effective alternatives to chemical pest control. To date deleterious effects on other components of the ecosystem have not been demonstrated. However, safety testing recommended for registration utilize protocols develope for chemical pesticides. Safety testing should respond to improving technology. The concensus of the symposium participants was that (1) safety testing protocols be modified such that they are appropriate for biological agents, (2) the sensitive and refined tools of molecular biology such as restriction fragment analysis, nucleic aci hybridization techniques, radioimmunoassay, etc., offer improved levels of specificit for virus identification and/or detection of virus replication, (3) the question of persistence or expression of viral genome or parts of viral genome in non-target systems should be of primary concern, (4) EPA confirm the data most relevant to safet tests before a biologic agent is registered, and (5) detailed studies on replication, pathogenesis and genetics of these agents in their natural hosts and cell culture systems should be carried out. These general recommendations emphasize only certain major topics urgently in need of research to provide basic information necessary for more precise and rational assessment of possible health effects of biologic agents used as pesticides.

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Viral Pesticides: Present Knowledge and Potential Effects on Public and Environmental Health

Symposium Proceedings

Edited by

Max D. Summers, Ph.D.
Department of Entomology
Texas A&M University
College Station, Texas 77843

and

Clinton Y. Kawanishi, Ph.D.
Health Effects Research Laboratory
Toxic Effects Branch
U.S. Environmental Protection Agency
Research Triangle Park, North Carolina 27711

Health Effects Research Laboratory
Office of Health and Ecological Effects
U.S. Environmental Protection Agency
Research Triangle Park, North Carolina 27711

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Foreword

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our nation's population.

The Health Effects Research Laboratory, Research Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis, and the toxicology of pesticides as well as other chemical pollutants. The Laboratory develops and revises air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is preparing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

The majority of the registered pesticides are chemical agents. A few, however, are biological in nature because the active ingredients are microbial. Of these microorganisms, viruses are perhaps the most unique in structure, biology, and the intimacy of their parasitic relationship with their hosts. These proceedings consider whether potential biohazards to human health and other biological components of the environment exist when insect viruses are used as pesticides and whether such potentials have been adequately assessed in view of our current knowledge of these agents.

Gordon Hueter, Ph.D.
Director
Health Effects Research Laboratory

Preface

The time is appropriate for the development and implementation of alternative and/or supplemental methods to chemical pest control, and a variety of strategies are being considered in integrated pest management programs. A series of conferences since 1970 have documented that insect pathogenic viruses such as baculoviruses represent one of the most promising approaches in pest control strategies. A joint FAO/WHO meeting in 1972 on "The Use of Viruses for the Control of Insect Pests and Disease Vectors" evaluated those insect viruses with the best potential for safe use. From that conference a test protocol was prepared pertinent for safety testing of promising viral pesticide agents. Some of the general conclusions of the FAO/WHO conference were: 1) Adequate safety tests and precautions were essential not only to safeguard vertebrates and other nontarget animals, but to minimize the risks of unforeseen accidents that might prejudice future development and use. 2) Identification of insect viruses was required to determine the pattern of specificity. 3) There was need for routine and sensitive methods for assaying all stages of virus infection and for monitoring and precisely identifying the virus or viral agents responsible. 4) Research was needed on the specificity of infection and the defense mechanisms of invertebrates. 5) Safety testing should detect and determine whether or not a baculovirus could enter vertebrate cells. 6) Since genetic changes could occur not only in susceptible hosts but the virus, a means of evaluating mutability was needed. 7) Consideration was given to the potential risks for nontarget invertebrates and whether such risks may increase depending upon the host range of the viral agent and the extent to which it was used outside of its natural geographical distribution.

The major conclusions and recommendations went essentially unnoticed until 1974 when a joint EPA-USDA Working Symposium was convened on "Baculoviruses for Insect Pest Control, Safety Considerations." Selected scientists, including a few vertebrate virologists, were convened with insect virologists and scientists involved in applied studies to critically evaluate the problems related to the development and safe use of baculoviruses. The discussions and major recommendations of that conference echoed those published in the WHO/FAO publication of 1973. However, at that time the molecular biology of baculovirus structure, genetics, and infection processes had not advanced much relative to the state of the art in 1972. The reports and recommendations of that conference are particularly pertinent within the context of the discussions and recommendations of this symposium (M.D. Summers, R. Engler, L.A. Falcon, P. Vail, Baculoviruses for Insect Pest Control: Safety Considerations. American Society for Microbiology, 1975). As a result of the joint EPA-USDA conference, a Baculovirus Research, Registration and Development Program was organized, formally recognized, and accepted by the Environmental Protection Agency in 1975; because of a variety of problems it did not realize functional organization. The program was designed to identify and clarify research priorities and set up a mechanism for the development and safe application of baculoviruses as pesticides.

We convened again at Myrtle Beach, South Carolina, in 1977, under the auspices of the US EPA, Health Effects Research Laboratory at Research Triangle Park, to continue our dialogue of many of the same questions and problems related to this unique approach to pest control. However, this symposium differs from previous efforts in the focus and nature of the background and expertise of the invited participants. The primary intent of this symposium was the evaluation of insect viruses by plant, insect, and vertebrate virologists with expertise in epidemiology and molecular biology from basic and applied perspectives to ascertain whether, based on this knowledge, a pragmatic assessment can be made of the safety of these agents for use as pesticides.

We attempted to juxtapose the objective opinions of the plant and vertebrate virologists with those of insect virologists to scientifically and comprehensively evaluate the benefits, potential risks, and problems of such an approach. It is hoped that these proceedings will be a source of information and sound base to facilitate productive, organized activity on basic and applied research for the development and safe use of viral pesticides.

Max D. Summers, Ph.D.
Clinton Y. Kawanishi, Ph.D.

Abstract

A select panel of invertebrate, vertebrate, and plant virologists, each a recognized authority and representing specialized areas in the field of virology, was convened to review the progress and status of the development and use of insect viruses, in particular baculoviruses, as biological pesticides. In integrated pest management practices the concept and approach of using certain insect viruses as specific biological pest control agents have been shown to be effective and provide a promising alternative and/or supplemental approach for pest control practices. In fact, two such viruses have been registered by the Environmental Protection Agency for control of important insect pests of forest and cotton ecosystems.

Available data have not revealed any deleterious effects of virological pesticides on other invertebrates, plants, and vertebrates, including man, in the ecosystem. The Environmental Protection Agency has registered viral pesticides based on protocols for chemical pesticidal agents and on the assumption that by using viruses which occur naturally biohazardous situations will not arise. However, it was also recognized that with commercial development and/or the selective development of more effective and virulent viruses and some of the problems associated with patenting natural agents, such approaches will likely bring this group of viruses into the realm of genetic manipulation.

Also, the use of *in vitro* production systems to mass produce viral pesticides without appropriate quality control could result in undesirable

consequences. It is recognized that there is worldwide development of microbial agents for use as pesticides and consequently it may be necessary to implement more stringent supervision and regulatory practices in order to monitor for potentially hazardous combinations, e.g., the contamination of a viral pesticide by a vertebrate virus that may arise as a result of *in vitro* and *in vivo* production.

Safety testing and safety testing criteria were evaluated relative to the state of the art and knowledge of factors responsible for baculovirus specificity and infection of susceptible and nonsusceptible hosts. It was emphasized that knowledge of the molecular biology, pathogenesis, and genetics of insect viruses is not presently comparable to that in other areas of vertebrate virology. Therefore, the technology to use or assess invasion and infection of susceptible and nonsusceptible hosts needs to be improved. It was the general opinion that some of the most sensitive methods for detecting virus entry and replication in exposed cells or hosts should be incorporated into Environmental Protection Agency safety testing guidelines. Of major importance was the development and application of more sensitive and refined technology for identification and detection of virus or viral genomes. Consideration of the question of persistence or expression of viral genomes or part of genomes in permissive and non-permissive systems indicated that DNA probe technology and sensitive serological assays should be applied to a spectrum of baculovirus exposed cell types that is representative of organisms in the treated environment.

Because many specific and sensitive detection techniques to monitor insect viral activities in the physical or biological environment are presently available, it was recommended that the Environmental Protection Agency hasten implementation of specific recommendations for evaluating safety with improved virus identification procedures and sensitive methods for detecting virus invasion and/or infection of nontarget systems. The majority agreed that data most pertinent or relevant to evaluating the safe use of viral pesticides be reconfirmed by the Environmental Protection Agency. To provide a more effective and comprehensive mechanism for the continued evaluation of the progress and development of viral pesticides, it was emphasized that more basic research in the area of understanding the factors responsible for replication and pathogenesis of insect viruses in their natural host systems be studied. Basic to this are the study of the genetics of these viruses and the identification of specific genetic and phenotypic markers which can be used for more precise studies of the molecular biology and ecology. It was emphasized that the use of specific DNA probes for genetic markers and the identification of specific phenotypic markers which can be detected by such techniques as radioimmunoassay be given high priority.

It was specifically emphasized that procedures for safety evaluation of chemical pesticides are not adequate for biological agents such as viruses. The lack of a clear procedure and policy for the development and registration of viral agents relative to safety and regulatory acceptability causes considerable uncertainty. It was also noted, however, that flexible guidelines are needed because the area and its application could possibly develop so rapidly that it would be inappropriate to require specific safety testing procedures. Consequently, the use of viruses as alternative control agents is wrought with misunderstanding, misinterpretation, and so forth. Such an atmosphere discourages long-term planning and development. Furthermore, viruses are only one class of a large variety of potential biological pesticide agents and compounds which may be rapidly forthcoming for use in integrated pest management. The Environmental Protection Agency's scope and vision must encompass these as well.

It was recommended that an advisory committee of expert vertebrate and invertebrate virologists be immediately appointed, initially as an ad hoc committee and later as a permanent panel to provide a creditable base of opinion and information to evaluate present needs and provide an objective forum for continued considerations of potential biohazard relative to other living systems in the environment. The value of such a committee would be in situations where during the course of research virus-host interactions are discovered that may appear alarming but perhaps are placed out of context relative to risk and public safety. Such reports could have profound influence on public opinion. Much of this could be moderated or more carefully evaluated by having the appropriate advisory group to deal with such situations.

The development and use of biological agents for regulation and pest control may presently not be of such magnitude as to appear to be of major immediate importance. However, because of the problems associated with environmental pollution, persistence, toxicity, and the general welfare of public health, large-scale use of many major chemical pesticides may be curbed one or two decades hence. The use of biological agents or products within the framework of integrated pest management strategies is being considered in agriculture in the United States and in underdeveloped countries. Information presented at this conference indicates a significant increase of this kind of effort on a worldwide basis.

Program Committee

AUGUST CURLEY
Chief, Toxic Effects Branch
U.S. Environmental Protection Agency
Health Effects Research Laboratory
Environmental Toxicology Division (MD 66)
Research Triangle Park, North Carolina 27711

ROBERT R. GRANADOS, PH.D.
Virologist
Boyce Thompson Institute
Cornell University
Ithaca, New York 14853

W.K. JOKLIK, PH.D.
Department of Microbiology and Immunology
Duke University Medical Center
Durham, North Carolina 27710

CLINTON Y. KAWANISHI, PH.D.
U.S. Environmental Protection Agency
Toxic Effects Branch
Health Effects Research Laboratory
Environmental Toxicology Division (MD 67)
Research Triangle Park, North Carolina 27711

MAX D. SUMMERS, PH.D.
Department of Entomology
Texas A&M University
College Station, Texas 77843

VICTOR STOLLAR, M.D.
Professor of Microbiology
College of Medicine and Dentistry of New Jersey
Rutgers Medical School
Piscataway, New Jersey 08854

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Virology Panel

JEFFREY J. COLLINS, PH.D.
Assistant Professor of Surgery and
of Microbiology and Immunology
Duke University Medical Center
Post Office Box 2926
Durham, North Carolina 27710

RICHARD A. DICAPUA, PH.D.
Associate Professor of Immunology
School of Pharmacy
University of Connecticut
Storrs, Connecticut 06268

RETO ENGLER, PH.D.
U.S. Environmental Protection Agency
Toxicology Branch
Registration Division (WH-567)
401 M Street, S.W.
Washington, D.C. 20460

LOUIS A. FALCON, PH.D.
Associate Insect Pathologist
Department of Entomological Sciences
333 Hilgard Hall
University of California
Berkeley, California 94530

BERNARD N. FIELDS, M.D.
Professor of Microbiology and
Molecular Genetics
Chief of Infectious Diseases
Harvard University Medical School
Peter Bent Brigham Hospital
25 Shattuck Street
Boston, Massachusetts 02115

ROBERT R. GRANADOS, PH.D.
Virologist
Boyce Thompson Institute
Cornell University
Ithaca, New York 14853

KEITH A. HARRAP, PH.D.
NERC, Unit of Invertebrate Virology
5 South Parks Road
Oxford, OX1 3VB
UNITED KINGDOM

JOHN A. HOLOWCZAK, PH.D.
Associate Professor of Microbiology
Department of Microbiology
College of Medicine and Dentistry
of New Jersey
Rutgers Medical School
Piscataway, New Jersey 08854

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PEPPER HOOPS
Department of Entomology
Texas A&M University
College Station, Texas 77843

CARLO M. IGNOFFO, PH.D.
Laboratory Director
U.S. Department of Agriculture, ARS
Biological Control of Insects Research Laboratory
Post Office Box A
Columbia, Missouri 65201

W.K. JOKLIK, PH.D.
Chairman
Department of Microbiology and Immunology
Duke University Medical Center
Durham, North Carolina 27710

CLINTON Y. KAWANISHI, PH.D.
U.S. Environmental Protection Agency
Toxic Effects Branch
Health Effects Research Laboratory
Environmental Toxicology Division (MD 67)
Research Triangle Park, North Carolina 27711

DENNIS L. KNUDSON, PH.D.
Yale Arbovirus Research Unit
Yale University School of Medicine
60 College Street
New Haven, Connecticut 06510

JOHN F. LONGWORTH, PH.D.
Insect Virology Section
DSIR Entomology Division
Private Bag
Auckland, NEW ZEALAND

WILLIAM J. MCCARTHY, PH.D.
Associate Research Virologist
Pesticide Research Laboratory and Graduate Center
Pennsylvania State University
University Park, Pennsylvania 16802

WILLIAM MEINKE, PH.D.
Virologist
Department of Microbiology
College of Medicine
University of Arizona
Tucson, Arizona 85724

JOSEPH S. PAGANO, M.D.
Director, Cancer Research Center
University of North Carolina
Box 3, Swing Building 217H
Chapel Hill, North Carolina 27514

FRED RAPP, PH.D.
Professor and Chairman
Department of Microbiology
Pennsylvania State University
College of Medicine
500 University Drive
Hershey, Pennsylvania 17033

ROBERT E. SHOPE, M.D.
Professor of Epidemiology
Yale Arbovirus Research Unit
Yale University School of Medicine
60 College Street
New Haven, Connecticut 06510

RALPH SMITH, PH.D.
Department of Microbiology and Immunology
Post Office Box 3020
Duke University Medical Center
Durham, North Carolina 27710

VICTOR STOLLAR, M.D.
Professor of Microbiology
College of Medicine and Dentistry of New Jersey
Rutgers Medical School
Piscataway, New Jersey 08854

MAX D. SUMMERS, PH.D.
Department of Entomology
Texas A&M University
College Station, Texas 77843

LOY E. VOLKMAN, PH.D.
Microbiologist
Department of Microbiology
Lovelace-Bataan Medical Center
5200 Gibson Boulevard, Southeast
Albuquerque, New Mexico 87108

MILTON ZAITLIN, PH.D.
Professor of Plant Pathology
Department of Plant Pathology
Cornell University
Plant Science Building
Ithaca, New York 14853

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Recommendations

EPA Symposium: Viral Pesticides: Present Knowledge and Potential Effects on Public and Environmental Health. Myrtle Beach, South Carolina, March 21-23, 1977.

Baculoviruses provide a promising alternative approach to pest control. Current evaluation methods suggest that the viruses are effective. Furthermore, available data have not revealed any deleterious effects for other components of the ecosystem, i.e., other invertebrates, plants, and vertebrates including man.

Safety testing criteria should continually respond to improved technology. We draw attention to the "Guidance for Safety Testing of Baculoviruses" section B-1, b-2.* It is important that the most sensitive methods for detecting virus replication are incorporated into the EPA guidelines for safety testing of baculoviruses. Recent developments in molecular biology have provided more sensitive and refined tools and offer the potential for testing at improved levels of specificity. By their implementation, the opportunity can be taken to improve further the safety tests for baculovirus pesticides. We draw attention again to the identification criteria listed on page 179 in "Guidance for Safety Testing of Baculoviruses."* The recent development of restriction fragment analysis of a variety of DNA-containing viruses has provided a powerful new tool which should be included among the identification techniques.

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*Baculoviruses for Insect Pest Control: Safety Considerations. M.D. Summers, R. Engler, L.A. Falcon and P.V. Vail, eds. American Society for Microbiology, Washington, D.C., 1975. 186 pp.

A fundamental concern for safety requires investigation of the question of persistence or expression of the viral genome or parts of the viral genome in non-target systems. Such tests should include at least two human cell types, rodent, avian, and fish cells. The development of such tests should embrace a variety of techniques such as molecular hybridization and radioimmune assay.

Before a biologic agent is registered as a viral pesticide, the data most relevant to the safety tests should be reconfirmed by EPA.

We strongly advise that EPA move with all speed to implement the above recommendations for safety involving identification, baculovirus genome tests, and sensitive methods for detecting virus replication, at least by January 1979.

Research Program

To provide a continuing basis for improvement and an evaluation of new products, it is imperative that certain areas bearing on the safety of insect viruses, particularly baculoviruses, should be fully understood.

1. A better understanding is needed of the replication and pathogenesis of the viruses in their natural hosts and cell culture systems.
2. The genetics of the viruses and the development of specific genetic markers are vital for precise ecological study. For example, the possible range of interaction between the viral pesticide and other viruses in the biologic environment needs to be explored.

These general recommendations of the invited panel are intended to focus attention on areas urgently in need of research. They represent only certain elements of major issues raised in the panel discussion, and they are put forward as the basis for the formulation of a specific program at an early date. We feel that this could best be achieved by a permanent and independent advisory panel.

Introduction

Max D. Summers, Ph.D.
Texas A&M University
College Station, Texas

Historically, the major effort in the field of viral pesticides has been in agriculture. However, the application of viral pesticides has a potential effect on human welfare as well. Pest control in this context of using insect pathogens is not new. Viral pesticides have been used in both artificial and natural environments since the evolution of insect viruses. Various components of agriculture have merely attempted to exploit viruses in a pragmatic way for economic benefit. Biological agents and, in particular, viruses and fungi have been and currently are considered as alternatives or aids in pest management strategies for several reasons:

- The development of resistance and cross resistance to chemical pesticides
- The rising cost of chemicals and application technology
- Environmental pollution, which on a long- and short-term basis, has undesirable side effects related to public health.

Because of this, there is a justified interest in developing more natural or biodegradable compounds such as insect viruses.

Those of us working with viral pesticides have been through this subject many times before. I would like to cite a few conferences that have taken place over the past few years dealing with this area of interest. In 1973, there was a joint publication as a result of a 1972 WHO-FAO (Food and Agriculture Organization) conference on insect viruses and their use in biological control. In 1974, a joint EPA-USDA (U.S. Department of Agriculture) conference on baculovirus safety was convened in Washington. Also in 1974, there was a conference sponsored by the EPA on the impact of the use of microorganisms on the aquatic environment (Pensacola, Florida). In 1975, the United States National Academy of Sciences published a five-volume comprehensive evaluation entitled Pest Control: An Assessment of Present and Alternative Technologies. In addition, there have been numerous meetings with and within the Society for Invertebrate Pathology. In 1976, the USDA and the Agricultural Research Service (ARS) met as a working group to prepare a working document on biological control agents, and organized activity will develop from that. The result is that the problems and benefits of using viral pesticides have already been identified, and recommendations have been made by capable scientists.

Yet, as is always the case in a developing science, there is concern about whether adequate precautions have been made to predict or assure the safe use of viral pesticides. There are those who are confident that safety is not a problem, those who advise caution, and those who demand absolute criteria and procedures to measure and/or predict safe use of viral agents. We have learned from experience that despite all available knowledge of safe application for chemical pesticides, we can make serious, irreversible mistakes. These mistakes are usually unintentional, but they can endanger public health for generations to come. Therefore, it is not so unrealistic to give some organized thought to the safe use of viral agents, placing in perspective our knowledge of mechanisms of infection relative to available technology.

We approach our task at this conference with an awareness of the problems and potential problems that may or may not occur to endanger public health. Present estimates from all previous conferences and published information suggest that viral pesticides are safe to use, and that the potential application of viral agents as insect control agents is quite good. Historically, there has been more effort in this area than one might think. Presently, an intensified effort is directed toward the increased use of viral pesticides, and it is likely that much more effort will be expended in future programs.

I feel that this meeting is unique as compared to previous meetings. Here, the focus is on the virologist's perspective, relative to the application of viruses in their proposed role. We have been fortunate to assemble a few select individuals working in insect virology. But that group has met many times before and because of that, some may feel that this conference is a waste of time. Previously, however, the virological content of our conference has been included with discussions of applied and field studies. Although that area is represented at this conference, that emphasis is not the primary focus of this meeting. Our attention will be directed toward the basic science of invertebrate virology and whether we are adequately prepared with the proper tools and knowledge to assure the safe use of viral pesticides, or to predict possible situations that could cause undesirable consequences. This is our most important obligation because the practical use of viruses in pest management schemes is really going to evolve during the next two decades.

One of the major factors that makes this conference unique is that we have been able to include as participants several prominent vertebrate and plant virologists. I would like to thank those participants and also add that of the original 12 virologists Drs. Joklik and Schlessinger recommended, only two declined.

I would like everyone to be frank and straightforward in identifying any problems or benefits of viral pesticides research, taking a scientific attitude, and keeping politics at a minimum. By the end of this conference, we would like to identify some major points that will aid in the development of the basic science and application of viral pesticides and provide a list of recommendations and priorities. This should be accented from the virologist's perspective. With that as an introduction, I would like to turn the program over to the Environmental Protection Agency and Dr. Frode Ulvedal.

Frode Ulvedal, Ph.D.
Environmental Protection Agency
Washington, D.C.

As many of you are aware, viruses are just one taxonomic group of biological agents that is being developed, registered, and used as a pesticide. According to the philosophy propounded in the 1972 amendment of the Federal Pesticide, Fungicide, and Rodenticide Act, which states in Section 20 that the administrator of EPA will undertake research to grant or contract with other Federal agencies, universities, and others to carry out the purposes of the Act, and in the context of this symposium, to give priority to research to develop biologically integrated alternatives for pest controls -- our agency is encouraging the development of effective pest control strategies while charged with the responsibility to ascertain that the remedy is not worse than the original problem.

Although the biological agents we will discuss in this symposium are present as normal components of the environment, when they are used as pesticides they are comparatively new to the agency, and our knowledge in this area is still developing. Currently, according to my knowledge, only two baculoviruses are registered for use as pesticides, but many others are being considered. Consequently, we must continually update our knowledge of biological agents as information about their physical properties becomes available through our efforts to protect humans against their adverse effects on health. We hope this symposium will contribute to this objective.

Gathered here as speakers and panel members are scientists knowledgeable about invertebrate viruses. This is, therefore, a unique opportunity for the speakers to impart to their distinguished medical and plant virology colleagues on the virology advisory panel the present knowledge of invertebrate viruses, and the status of accumulated knowledge concerning the properties of medically and agriculturally important viruses. We seek their advice on whether they see potential problems associated with the use of viruses as pesticides, and their aid in determining the necessary research.

By providing information on the unique properties of viruses, the proceedings of this symposium should assist the Environmental Protection Agency in its efforts to develop more rational and relevant approaches to safety testing and registration. Additionally, the symposium should facilitate development of the viral agents to their full potential as a unique class of alternative pesticides that are effective, less disruptive to the environment, and hopefully, not detrimental to human health. I hope we shall all gain knowledge and a perspective of viruses as potential pesticidal agents from the ensuing presentations and discussions.

Victor Stollar, M.D.
Rutgers Medical School
Piscataway, New Jersey

I would like to make a few brief remarks before getting into the content of the program. Some of us feel ill at ease dealing with a large group of viruses that are largely unfamiliar to us. I hope this uneasiness will be dispelled shortly. I realize also that we should keep politics out of our discussions. Nevertheless, I was struck by a front page newspaper article several months ago that quoted a Senate staff report describing the government program for assuring the safety of pesticides as chaotic. This article referred, fortunately, to chemical pesticides. I assume that part of our task at this conference is to see that viral pesticides will be used in the best possible way.

I think Dr. Summers has already formulated the basic problems we wish to address. Just to run through them again, the questions we want to keep in our minds over the next few days are: "Is the use of viral pesticides feasible? If so, which viruses can be used?" We want to keep in mind what we know about these viruses and what we still have to learn about them. We need solid, basic, and theoretical information, as well as which techniques in the laboratory and in the field need further development. We want to come out of this meeting with recommendations, both general and specific. I think these recommendations will come out of the discussion panels. We also want to keep in mind the potential hazards of using viral pesticides.

The presentations during the first part of the day are going to be formal and informative; we would prefer minimal discussion or questions following them. I think the most appropriate kinds of questions would be those that are strictly informational or questions to clarify unclear material. Let us leave the pertinent discussion for the latter part of the afternoon.

Not all of the participants are listed on the formal program, but certainly, we want and need the advice, suggestions, and criticisms of all the participants. We are going to hear first about the present status of viral pesticide programs, both in this hemisphere and in international programs. These are large-scale programs.

I do not want to steal attention from the first two speakers, but I thought it might be interesting to draw to your attention a magazine article. I like to work in the garden on weekends, and for several years, I have had a subscription to a journal called Organic Gardening. I was quite struck last October to see an article called "An Insect Control Method Too Good To Be True." The article describes, basically, a very simple method of insect control for the do-it-yourself gardener, using insect viruses. The article describes a process of finding dead and sick insects, homogenizing them, diluting them, and spreading them in the garden. So, certain elements in the field of viral pesticides may, in a sense, be already out of control.

PART I

CURRENT USE OF VIRUSES AS ALTERNATIVES TO CHEMICAL PESTICIDES

Viruses as Alternatives to Chemical Pesticides in the Western Hemisphere

Louis A. Falcon, Ph.D.
Division of Entomology and Parasitology
University of California, Berkeley

Chemical pesticides are overpromoted and overused throughout most of the Western World. From personal experiences, I would say that more than half the pesticide used in agriculture is not necessary and is wasted. Recognizing that indiscriminate use of pesticides is a contributing factor in environmental pollution, more people are becoming interested in creating and maintaining a healthy environment with little or no chemical pesticides. As an agricultural entomologist by training and an environmentalist out of necessity, I am working with others in an effort to find and develop satisfactory alternatives to chemical pesticides. Several directions are being followed, one of which is the development of microbial agents for pest control. In this conference we deal specifically with the development of one group of microbial agents, the insect viruses.

Of the known insect viruses, the nuclear polyhedrosis (NPV) and granulosis viruses (GV) of the genus Baculovirus continue to offer the greatest potential for insect pest control in agriculture and forestry. They appear particularly well suited for this purpose because candidate viruses tested thus far have demonstrated:

1. Efficacy and usefulness in the control of target insect pests.
2. A high degree of specificity, which makes them especially valuable for use in integrated pest management programs.

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3. Safety, as they have never been shown to be hazardous to humans or other warm-blooded animals.

Although this conference will examine various types of insect viruses, my comments apply specifically to the baculoviruses.

TO WHAT EXTENT ARE INSECT VIRUSES BEING USED AS ALTERNATIVES TO CHEMICAL PESTICIDES IN THE WESTERN HEMISPHERE

There has been and continues to be little direct use of insect viruses for pest control in the Western Hemisphere. In the United States only two insect viruses have been registered with the EPA and are available for commercial use. One is the product Elcar®, which contains the NPV of the bollworm, *Heliothis zea*. It was registered by Sandoz, Inc. The other insect virus product was registered by the U.S. Forest Service, and contains the NPV of the Douglas fir tussock moth (*Orgyia pseudotsugata*). A third baculovirus, the gypsy moth (*Porthetria dispar*) NPV, is close to registration. Only one other baculovirus is receiving sufficient attention at this time to be considered within the range of registration and that is an NPV originally isolated from the alfalfa looper, *Autographa californica*. Over the past two years the Agricultural Research Service (ARS) of the U.S. Department of Agriculture has made efforts to develop a petition for an experimental use permit and an exemption from a tolerance for this NPV from the EPA (A.M. Heimpel, personal communication). I am not aware of efforts to register insect viruses in other countries of the Western Hemisphere with the exception of Canada where scientists are working closely with their U.S. counterparts on the same viruses.

In 1976, Sandoz, Inc., began marketing Elcar in several cotton growing states of the United States. While only small quantities were reported sold during the first year, the market is expected to increase as growers become aware of the availability of this selective microbial control agent. Research is continuing in an effort to improve the Elcar product with much attention being given to formulation and methods of application. This particular product has provided a model for the development and commercialization of other baculovirus pest control products.

So far the Douglas fir tussock moth NPV product appears to be little more than a curiosity on the shelf. There is a very real need for an educational program to train forest workers on the large-scale use of this baculovirus (1). Ostensibly, potential users would rather spend their energies trying to restore DDT instead of learning to use the Douglas fir tussock moth NPV.

During the last three decades, several baculoviruses have been employed for insect pest control, including the alfalfa caterpillar (Colias eurytheme) NPV for control of caterpillars on alfalfa in California during the 1950's (2); the NPV of the cabbage looper (Trichoplusia ni) which has been employed with good success by vegetable, cotton, and soybean growers in the U.S., Canada, Mexico, Nicaragua, and Colombia; the NPV of the beet armyworm (Spodoptera exigua) used in California and Arizona on cotton, alfalfa, and vegetables; and NPVs isolated from various species of sawflies (Neodiprion and Diprion spp.) for the protection of Christmas tree plantations and forests in the U.S. and Canada.

WHAT IS THE POTENTIAL FOR USING INSECT VIRUSES AS PEST CONTROL AGENTS

One way to examine the potential for using insect viruses as pest control agents is to look at agricultural crop loss figures for various insect pests which can be controlled by these agents. Let us look at pest losses in California (Annex A). This is the leading agricultural state in the U.S., growing nearly 50% of the fruits and vegetables consumed in the nation. In addition, it is the top cotton producing state, and this industry is the major consumer of chemical pesticides in the U.S. In 1974, agricultural returns for California amounted to \$8.5 billion, or about 11% of the total agriculture product for the United States, which was \$95 x 10^9.

In Central America, Nicaragua imported over US\$ 10 x 10^6 in chemical pesticides to protect a cotton crop worth US\$ 60 x 10^6 in 1973. The results of a 3-year study showed that biological control agents including the use of insect viruses could reduce expenditures for pesticides by 50%. That is a large financial saving for a developing country with an annual total government budget of about US\$ 85 x 10^6 (3).

Taking the Western Hemisphere as a whole, it appears that about 30% of the current insect pest problems in agricultural crop production are susceptible to control by insect viruses, specifically the baculoviruses. The potential economic benefits from their development and use are significant.

WHAT IS THE CURRENT INPUT FOR DEVELOPING INSECT VIRUSES FOR MICROBIAL CONTROL

Overall, the current yearly expenditure devoted to the research and development of insect viruses appears to be circa US$ 5 x 10^6 for the entire Western Hemisphere. This is equivalent to about 2.5% of the estimated overall crop loss caused by virus susceptible pest species for California alone. On a hemisphere basis it is probably less than .0025%. Most of the research and development activity is in the U.S. and Canada with scattered activity in a few South American countries, namely Colombia, Venezuela, and Nicaragua. Less than 60 scientific man years are currently going into viral pesticide research and development each year.

For the United States, the data I compiled indicated that the current yearly investment is about $1.6 million at the state level, i.e., for agricultural experimental stations and universities. Only eight states are involved in insect virus pest control research, and California appears to have the biggest commitment with about a $300,000 yearly investment (Annex B). At the Federal level, the USDA is investing about $2.0 million yearly. Six ARS and two FS stations are doing the bulk of the research and development work. Recently it was reported that 75% of the USDA's pest control research budget is for support of nonchemical pesticide research. If this is true, apparently less than 1% of this money is used for insect virus pesticide research and development. In the commercial arena, industry appears to be investing less than $400,000 per annum. In recent years only Sandoz, Inc., and Nutrilite Products, Inc., have been actively involved in developing insect viruses for pest control. The problems associated with the development and industrialization of insect viruses were fully discussed by Falcon (4).

WHAT IS THE EFFORT NEEDED

In 1973, the Food and Agriculture Organization (FAO) of the United Nations published a list of the NPVs and GVs (Annex C) that would be useful for integrated pest control (5). While this list was developed on a global basis, it applies equally well to the Western Hemisphere. Under the category of most promising insect viruses needed for integrated pest control, a maximum of 12 NPVs and a minimum of two NPVs are indicated. The former figure assumes one NPV per insect species listed in categories 1, 2, and 3 and one for category 4, while the latter takes into account the broader host range of some NPVs such as Autographa NPV, which can infect several species of lepidoptera. In addition, four GVs were listed. The list encompasses the major lepidopterous pest species of food, fiber, and oil crops in the world.

Along with the above, another category -- "... viruses that may have good potential for commercial production for integrated pest control" -- was also included. Five NPVs and two GVs were listed here. In conclusion, according to the FAO list, a maximum of 16 NPVs but possibly as few as seven NPVs plus six GVs are all that are needed to achieve high utilization of insect viruses for pest control in the Western Hemisphere and, for that matter, on a worldwide scale.

As demonstrated with the Heliothis and Douglas fir tussock moth NPVs, the technology is available to produce, formulate, and store sufficient quantities of each insect virus for small- and large-scale field testing purposes. A protocol for safety testing has been published (6). Also, an EPA publication providing guidelines to aid in determining the efficacy and usefulness of candidate baculoviruses is being printed and should be available in 1978.

The case I have attempted to develop shows that the total effort needed to develop insect viruses for pest control is not large, yet the rewards can be plentiful. I calculated that an additional US$ 1.6 million per year for five years is required to undertake the basic research and development required to make the 16 insect viruses listed in category 1 (Annex C) available for pest control. Ideally the monies should be coordinated through a central agency, such as the National Institutes of Health (NIH) or National Science Foundation (NSF), and made available to qualified and interested researchers through competitive grants subjected to peer review. The bulk of the monies should be allocated to efficacy testing, as this activity will select the useful insect viruses which can then be subjected to complete safety testing for registration purposes.

A model to follow for developing the program described above is a forest pest program involving the U.S. Forest Service and several universities (7). It deals with three pests: the Douglas fir tussock moth, the southern pine beetle, and the gypsy moth. The Federal funds that were made available through this program have permitted the development and registration of the Douglas fir tussock moth and the gypsy moth NPVs. There has never been a comparable program for agriculture.

WHY HAS SUPPORT NOT DEVELOPED

Support for the research and development of insect viruses has not developed mainly because naturally occurring arthropod viruses are not patentable (4). Thus there is little incentive for private industry to invest money for research and development as is done for chemical insecticides which are patentable. Furthermore, insect viruses do not have the market potential of chemical pesticides. Since they are selective and do not disrupt the environment, the use of insect viruses does not create the "treadmill effect" which often occurs with the use of chemical pesticides. The more chemical pesticides are used, the more they are needed, whereas insect viruses are needed less with increased use. Consequently, not only is there little incentive for private industry to develop insect viruses, they will do everything possible to prevent the development because they fear the competition.

Within USDA-ARS, the development of insect viruses has been hampered by competition from research on other control methods. For the past decade, the bulk of the pest control research dollar has been invested in the research and development of the sterile-male technique, an approach which failed to develop as anticipated.

Another reason why insect viruses have not developed rapidly is due to the rather hard position taken first by the Food and Drug Administration (FDA) and later by EPA. The policy is that insect viruses must be registered as pesticides in the same way as chemicals. The primary impact of this ruling was to stop the use of insect viruses for pest control in those areas where they were being successfully utilized. In actuality, insect viruses are naturally occurring components of the environment, the same as parasites and predators, pest insects, nematodes, bacteria, etc. Recently the EPA Registration Division agreed to permit the use of a wood-rotting fungus that when used as an inoculum on freshly cut tree stumps would not require registration because it is a "naturally occurring fungus." This has occurred with other agents, and demonstrates that the registration system is capable of some flexibility. However, everything possible must be done to ensure that this approach is applied correctly and fairly in all situations dealing with naturally occurring agents.

Another problem interfering with the development of baculoviruses has been and continues to be the "hazard monger." Hazard mongers are research workers who are also opportunists and see this as an area which can be developed to their advantage. They employ scare tactics about the hazards associated with viruses. They lump the viruses associated with insects and make broad generalizations, forgetting that the baculoviruses are really quite different from other insect viruses. What is needed is an objective, coordinated effort to demonstrate the safety of each virus to be used and to show why the baculoviruses are not hazardous (6).

A strong education effort is required to place the insect viruses in their proper perspective at all levels (i.e., lawmakers, regulatory agencies, researchers, users, and society in general). At the government regulation level, protocols and policies for registration and use must be established on a permanent basis, so these will remain, regardless of the "people-exchange" that goes on in Washington. Some progress has been made in this area as exemplified by the guidance for safety testing published by the EPA (6) and the EPA guidelines for establishing the efficacy and usefulness of microbial agents for pest control (in press).

Finally, the most difficult problem facing the development of insect viruses for pest control continues to be insufficient funding. Although a few research programs include insect viruses as part of their activities, the total effort is very small. Microbial control has never had full-fledged representation at decision-making levels of Federal programs. For example, there is no one representing this area on the National Program Staff of USDA on a full-time basis. Traditionally, it is an area assigned to others who are experts on something else.

In summary, I have attempted to show that the insect viruses, particularly baculoviruses, have potential as pest control agents and are sorely needed for this purpose. Only a very few, 16 or less, are needed to help combat about 30% of the insect pests in the field. The technology to develop them is available, but current efforts are minimal and haphazard at best. A strong national program with adequate funding is required to stimulate research and development. The capacity, technology, ability, and justification are there. At this point, it requires the right political moves to bring it about.

If this area is allowed to develop to its effective maximum level, the potential effects on public and environmental health will be beneficial. The possible hazards that may exist will be identified, and we will begin to understand why baculoviruses behave as they do. Most important of all could be the development of effective, useful, environmentally safe products which can replace the hazardous, environmentally unsuitable products that are in use today.

ANNEX A

ESTIMATED CROP LOSSES CAUSED BY INSECT SPECIES SUSCEPTIBLE TO CONTROL WITH BACULOVIRUSES IN CALIFORNIA DURING 1974*

Insects	Estimated overall loss
Alfalfa caterpillar - Colias eurytheme	$ 4,423,807
Alfalfa semilooper - Autographa californica	1,985,700
Armyworms - Pseudaletia unipuncta	
Prodenia praefica	47,862,147
Spodoptera exigua	11,236,695
Cankerworms - Alsophila pometaria	
Palecrita vernata	400,900
Codling moth - Laspeyresia pomonella	2,788,490
Corn earworm - Heliothis zea	69,695,996
Cutworms complex - Agrotes ipsilon (plus 4 other spp.)	10,615,424
Fruit-tree leafroller - Archips argyrospilus	3,708,708
Imported cabbageworm - Pieris rapae	966,775
Loopers - Cabbage - Trichoplusia ni	12,198,875
Other	1,149,902
Diamond back moth - Plutella xylostella	533,399
Oriental fruit moth - Grapholitha molesta	4,255,290
Pink bollworm - Pectinophora gossypiella	19,016,839
Potato tuberworm - Phthorimaea operculella	1,359,360
Tent caterpillars - Malacosoma spp.	296,900
Total	$192,495,207

*Source: Hawthorne, R.M. (8).

ANNEX B

ESTIMATED EXPENDITURES FOR BACULOVIRUS PEST CONTROL ORIENTED RESEARCH AND DEVELOPMENT AT PRINCIPAL LOCATIONS IN THE WESTERN HEMISPHERE FOR 1975-76

Location	Principal scientific man years*	Approximate expenditures†	
U.S.			
States - Agricultural Experiment Stations			
Alabama	1	$100,000	
Arkansas	2	200,000	
California	3	300,000	
Florida	2	200,000	
Mississippi	1	100,000	
Ohio	2	200,000	
Pennsylvania	2	200,000	
Texas	2	200,000	
			1,500,000
Federal - USDA			
ARS			
Beltsville, MD	2.5	250,000	
Brownsville, TX	1	100,000	
Columbia, MO	2	200,000	
Fresno, CA	1	100,000	
Phoenix, AZ	1	100,000	
Tifton, GA	1	100,000	
FS			
Corvallis, OR	6	600,000	
Hamden, CT	6	600,000	
			2,050,000
Private Industry			
Sandoz	3	300,000	
Nutrilite	.5	50,000	
Other Groups			
Boyce Thompson Institute	3	300,000	
			650,000
TOTALS - U.S.	42		4,200,000

(Continued)

ANNEX B (CONTINUED)

Location	Principal scientific man years*	Approximate expenditures†	
Canada			
Sault Ste. Marie	3	300,000	
Forestry Service	3	300,000	
Dept. of Agriculture	1	100,000	
			700,000
Colombia, S.A.			
Federacion Nacional de Algodoneros	.5	50,000	
Venezuela, S.A.			
Venezuelan Institute of Scientific Research	1	100,000	
			150,000
TOTALS - Western Hemisphere	50.5		US$5,050,000

*Calculations based on percent time allocated by each individual and rounded to nearest whole number in most cases.

†Estimated expenditures based on salaries, supplies and expense and equipment and facilities with $100,000 per scientist used as a mean figure.

ANNEX C

I. Most promising insect viruses needed for integrated pest control[*]

  A. Nuclear polyhedrosis viruses (NPV)

    1. Viruses of the *Spodoptera* complex (includes most species formerly in *Prodenia*, e.g., *littoralis*, *exigua*, *frugiperda*, *litura*, and *exempta*). At present includes a large complex of viruses. Hopefully, one microbial product would be effective against the entire complex.

    2. Viruses of the *Heliothis* complex, e.g., *armigera*, *zea*, and *virescens*. Hopefully, one product would be effective against the entire complex.

    3. Viruses of Plusiinae, e.g., *Trichoplusia*, *Plusia*, and *Pseudoplusia*.

    4. *Autographa californica* virus, which is cross-infective to genera in several lepidopterous families, e.g., *Pectinophora*, *Bucculatrix*, *Heliothis*, *Trichoplusia*, *Spodoptera*, *Estigmene*, and *Plutella*.

  B. Granulosis viruses (GV)

    5. *Laspeyresia pomonella* (codling moth).

    6. *Phthorimaea operculella* (potato tuberworm).

    7. *Pieris* (*Mamestra*) spp. (crucifer caterpillars).

    8. *Argyrotaenia velutinana* (redbanded leafroller).

II. Other insect groups with viruses that may have good potential for commercial production for integrated pest control

    9. *Neodiprion* complex (NPV).

    10. *Malacosoma* complex (NPV).

    11. *Agrotis*, *Peridroma*, and related cutworms (NPV).

    12. *Porthetria dispar* (NPV).

(Continued)

ANNEX C (CONTINUED)

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13. *Chilo suppressalis* (GV).
14. *Ephestia cautella* (NPV + GV).
15. *Mamestra brassicae* (NPV).

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*Source: FAO (5).

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DISCUSSION

SHOPE: Dr. Falcon, you covered very clearly the insects of agricultural importance, but you did not mention the need for baculovirus pesticides to control insects or arthropods of medical importance. Was this left out purposely, or do you see a need there also?

FALCON: I left this out purposely because the information I have received from people working in this area indicates there appears to be little potential in the area at this time. So far, viruses that have been isolated from insects of medical importance such as mosquitoes do not appear to be very virulent or effective. In general the application of insect pathogens against arthropods in this group has not been too successful. As I saw it, my responsibility was to review current programs. To my knowledge, there are no programs in this particular area. Possibly other people here might be able to comment further on this matter.

RAPP: You mentioned a 200 million dollar loss to agriculture in California due to insects susceptible to control with baculoviruses. How accurate are these figures?

FALCON: This type of information has been accumulating in California for about two decades. In 1970, for example, the same group of insects was estimated to have cost approximately 70 million dollars, so the increase has more than quadrupled in four years. Of course these types of data are susceptible to many variables and sometimes may be misleading, but they are the best we have. Also I wish to indicate that California is an ideal area for agriculture, and generally speaking, there is not the magnitude of pest problems that exists in other areas of the U.S. or, for example, in Central America. California is principally a desert-type agricultural situation and has fewer pest problems than many other areas. So the crop loss figures cannot be applied across the United States.

The International Scope of Invertebrate Virus Research in Controlling Pests

Keith A. Harrap, Ph.D., and T. W. Tinsley, Ph.D.
NERC, Unit of Invertebrate Virology
Oxford, United Kingdom

For convenience this survey has been divided into three sections:

1. Past, present, and future control programs.
2. The trend of research toward the goal of producing a viral pesticide and using it responsibly.
3. The problems associated with the irresponsible use of viruses as pesticidal agents.

The preceding paper was concerned with the situation in North America and the Western Hemisphere generally, and these geographical areas therefore are excluded from this presentation. Our comments here are limited to considering mainly Baculoviruses for pest control purposes.

For convenience, past and present control programs have been summarized by geographical area in Table 1 (Europe and Africa) and Table 2 (the Far East and South Pacific). Table 3 summarizes possible future virus control programs. These lists should not be considered exhaustive, and it is quite likely that there are or have been a number of small virus control projects against pest insects that are not represented. Let us now examine the various programs itemized in the tables in more detail.

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TABLE 1. VIRUS RELEASE PROGRAMS IN EUROPE AND AFRICA

Country or region	Insect host	Virus	Virus characterized	Safety tests	Application method	Reference
United Kingdom	Neodiprion sertifer (Hymenoptera)	Baculovirus - nuclear polyhedrosis	In progress	No*	Spray	(1, 2) Entwistle, Harrap & Robertson, (unpublished)
United Kingdom	Gilpinia hercyniae (Hymenoptera)	Baculovirus- nuclear polyhedrosis	In progress	No	Limited point source inoculum (dead insects)	Entwistle (unpublished)
Continental Europe	Lymantria monacha (Lepidoptera)	Baculovirus - nuclear polyhedrosis	In progress	No	Spray	Zethner (unpublished)
Continental Europe	Lymantria dispar (Lepidoptera)	Baculovirus - nuclear polyhedrosis	In progress	No	Spray	(3, 4)
Continental Europe	Thaumetopoea pityocampa (Lepidoptera)	Cytoplasmic polyhedrosis virus	No	No	Aerial dust	(5)
Continental Europe (Denmark)	Agrotis segetum (Lepidoptera)	Baculovirus - nuclear polyhedrosis	In progress	No	Spray	Zethner (unpublished)
Africa (Kenya)	Spodoptera exempta (Lepidoptera)	Baculovirus - nuclear polyhedrosis	Completed (1976)	In progress	Spray	(6, 7)
Africa (Egypt)	Spodoptera littoralis (Lepidoptera)	Baculovirus - nuclear polyhedrosis	Completed (1976)	In progress	Spray	(8, 9)
Africa (Uganda)	Heliothis armigera (Lepidoptera)	Baculovirus - nuclear polyhedrosis	In progress	No	Spray	(10)
Africa (South Africa)	Kotachalia junodi (Lepidoptera)	Baculovirus - nuclear polyhedrosis	No	No	Spray	(11, 12)

*Safety tests on this virus have been done by the U.S. Forest Service, Hamden, CT.

TABLE 2. VIRUS RELEASE PROGRAMS IN FAR EAST AND SOUTH PACIFIC

Country or region	Insect host	Virus	Virus characterized	Safety tests	Application method	Reference
Malaysia (Sabah, Sarawak)	Darna trima (Lepidoptera)	1. Baculovirus granulosis	In progress	No	Spray	(13)
		2. Small RNA viruses	Completed 1976	No		
Japan	Dendrolimus spectabilis (Lepidoptera)	Cytoplasmic polyhedrosis virus	?	Yes	Spray	(14) (15)
Australia	Pieris rapae (Lepidoptera)	Baculovirus granulosis	No	No	Spray	(16)
New Zealand	Pieris rapae (Lepidoptera)	granulosis	No	No	Spray	(17)
South Pacific	Oryctes rhinoceros (Coleoptera)	Baculovirus Oryctes virus	Completed 1975	?	Release of inoculated adults	(18, 19)

TABLE 3. POSSIBLE FUTURE VIRUS CONTROL EXPERIMENTS

Country or region	Insect host	Virus	Characterization	Safety tests	Probable method of application
United Kindgom and Continental Europe	Mamestra brassicae (Lepidoptera)	Baculovirus nuclear polyhedrosis	In progress	Projected	Infected insects Localized spray
Europe (Denmark, German Federal Republic)	Agrotis segetum (Lepidoptera)	Baculovirus nuclear polyhedrosis	In progress	?	Spray
United Kingdom	Neodiprion sertifer (Hymenoptera)	Baculovirus nuclear polyhedrosis	In progress	Projected*	Localized spray
Middle East (UAR, Morocco)	Spodoptera littoralis (Lepidoptera)	Baculovirus nuclear polyhedrosis	Completed (1976)	In progress	Spray
East Africa (Kenya)	Spodoptera exempta (Lepidoptera)	Baculovirus nuclear polyhedrosis	Completed (1976)	In progress	Spray
Seychelles	Oryctes monoceros (Coleoptera)	Baculovirus Orycetes virus	Completed (1975)	?	Release in infected adults

*Safety tests on this virus have been done by the U.S. Forest Service, Hamden, CT.

Two viruses have been used in the United Kingdom -- both against sawflies. With Neodiprion sertifer a number of ad hoc trials were made in Eastern England and Scotland around 1960 with crude preparations of larval cadavers (1, 2). Little quantification was possible as no virus purification was attempted, and the properties or even content of the spray application were not known in detail. This ad hoc type of spray application then ceased until characterization of the virus could be undertaken. This work is now nearing completion in our laboratory at Oxford. Application trials of purified virus start this year on a selected area of pine sawfly infestation in Scotland. N. sertifer virus is produced commercially in Finland and has also been used there for control of pine sawfly.

In Wales, areas of spruce were attacked by another sawfly, Gilpinia hercyniae, and a virus epizootic became established naturally. Only point sources of inoculum were used for further virus release so that virus spread could be monitored. A period of four years' observation of this epizootic by P.F. Entwistle and his colleagues from our laboratory has produced valuable data on the factors influencing the progress of a virus epizootic in a field situation.

Certain biochemical characteristics of nun moth (Lymantria monacha) and gypsy moth (Porthetria dispar) NPVs have been studied in Oxford by O. Zethner while he was a visiting worker in our laboratory. The nun moth NPV has been used in Denmark by Zethner in the Silkeborg North Forest in control projects. An area of about 15 hectares of forest was treated using virus purified by centrifugation and applied at 10^7 polyhedra per ml, 80 liters per hectare; 0.4% pinolene was used as a sticking agent. Approximately 90% mortality occurred as a result of NPV infection and half the larvae died as pupae. Damage in 1973 could not be avoided in the NPV-treated area, but during 1974 the population of the nun moth, as well as the damage caused, was very much reduced in the virus treatment areas in comparison with other areas of the forest both untreated and chemically treated (O. Zethner, personal communication). Detailed description of this work is presently in the course of publication.

Some very promising trials have also been done in Denmark by Zethner on cutworms (Agrotis segetum) using granulosis virus (GV). This work is in progress at present. Virus was produced in larvae reared on synthetic diet, purified by centrifugation, and applied in suspension. Approximately 80% reduction in damage to red beets and carrots was observed when compared with untreated control plots. There is some evidence that the virus remains effective one year or more after application.

In Africa Baculoviruses have been used against two species of Spodoptera. The properties of these viruses (and S. frugiperda and to a lesser degree S. exigua virus) have been fully investigated in Oxford, and the data are presently either in press or published (20, 21). There is also a considerable effort being made in our laboratory to understand the replication of these viruses in cell culture systems. Both viruses have been toxicity-tested (following the procedure recommended by the Insect Pathology Laboratory, USDA, as published in WHO Tech. Rep. No. 531) at the Microbiological Research Establishment, Porton Down, Salisbury, U.K. This work was contracted from the U.K. Ministry of Overseas Development, Centre for Overseas Pest Research, and this organization intends to evaluate these viruses in control programs in Africa and the Mediterranean. Purified virus is supplied by our laboratory for this work. In addition, tests for virus replication in those animals used in the toxicity testing procedures are now being undertaken by Oxford staff working at Porton Down. Briefly, the protocol here involves the inoculation of virus-sensitive cell cultures with tissue homogenates from animals subjected to the various routes of baculovirus exposure (e.g., oral, dermal, pulmonary). Preliminary results appear to demonstrate the ability of the cell culture systems to yield positive CPE with polyhedra formation from such types of inoculum. These viruses then are fairly well characterized and safety-tested. Application in the field is now the responsibility of the Ministry of Overseas Development, and they are also undertaking larval bioassay tests in preparation for field control projects. Under similar arrangements characterization work is in progress at Oxford for the baculovirus of the bollworm, Heliothis armigera, and Heliothis zea virus has been included in the program for comparison.

Table 2 shows similar information for the Far East and South Pacific. The first example, control of the oil and coconut palm pest Darna trima, will be considered in detail below. The CPV used in Japan apparently has been toxicity safety-tested but we have no details at present of the procedures used (22). We are not aware of safety testing or published data on the properties of the P. rapae granulosis viruses used in Australia and New Zealand. Oryctes virus has been used very successfully to control the beetle Oryctes rhinoceros in Samoa, the Philippines, and Malaysia and could possibly be used in the Seychelles to control Oryctes monoceros. This virus was characterized at Oxford at the request of FAO and we understand that it has been safety-tested in France but are not aware of the procedures used (23). Possible future control programs using viruses are given in Table 3. Several are continuations of programs already discussed and listed in Tables 1 and 2.

There have been many other pesticidal virus release programs in the USSR and Eastern Europe of which we have only scant information so a tabulation has not been attempted. However, a list of past and present preparations of viruses in or near commercial use (Table 4) shows three products from the USSR in addition to the more familiar North American products.

Clearly, then, there is a fair range of activity. Unfortunately, the state of the art is very different in the different examples cited. An evaluation of the trend of the research effort and responsible use of virus pesticides can therefore form a useful second part of this article.

In some cases (e.g., *S. littoralis*; *S. exempta*; *N. sertifer*; *H. armigera*; *H. zea*; *L. monacha*; *L. dispar*), a logical development is discernible toward the goal of using a *Baculovirus* as a pesticide. In other cases only certain parts of the work which we consider to be necessary are completed or even contemplated.

First, one should have knowledge of the physical, chemical, and biological properties of any virus that might ultimately find itself on the market place in the hands of the inexperienced operator. One can argue how complete such knowledge has to be, but certainly sufficient data on the virus should be available for it to be recognized; that is, identified as a specific candidate virus. Table 5 summarizes certain properties of *Spodoptera* NPVs which have enabled us to achieve this with these viruses at Oxford. Second, some safety testing of the purified and characterized candidate virus should be done for toxicity and more importantly for replicative events in nontarget cells and tissues. Accurate detection and recognition of the virus is a necessary prerequisite for assessing such tests. One can, of course, argue about the extent of such tests for fundamentally similar viruses. For example, how different in properties do the viruses have to be to justify individual safety tests? Third, knowledge of the replicative processes of *Baculoviruses* using established laboratory model systems could indicate any potential risk of a more subtle nature which large-scale application of these viruses might create. At some stage before safety testing has proceeded very far, or indeed even started, but after knowledge of the virus properties is available, one needs to demonstrate the effectiveness of the purified virus as a control agent in small field tests. In the U.K., registration under the Ministry of Agriculture, Fisheries and Food, Pesticide Safety and Precaution scheme is required to allow a trial of this type under strict conditions. This procedure is to be used this year by our laboratory in Scotland in a control trial using *N. sertifer* NPV. The virus characterization is virtually completed, control

TABLE 4. PAST AND PRESENT VIRUS PESTICIDES IN OR NEAR COMMERCIAL USE

Target insect	Virus type	Country of origin	Manufacturer	Trade name
Autographa californica	NPV	USA	USDA Beltsville, MD**	-
Choristoneura fumiferana	NPV	Canada	Canadian Forestry Service† Sault Ste. Marie	-
Dendrolimus spectabilis	CPV	Japan	Chugai Pharmaceutical Co. Ltd. & Kumiai Chemical Industry Co. Ltd.	Matsukemin
Heliothis zea	NPV	USA	Nutrilite Products Hays-Sammons Sandoz-Wander	Biotrol VHZ Virex Viron/H Elcar*
Hyphantria cunea	NPV	Japan	Kanagawa-ken Sericultural Center & Tokyo-to Sanshi Shidosho	-
Lymantria sp.	NPV	USA (*P. dispar*)	U.S. Forest Service, Hamden, CT**	-
		USSR	Glavmikrobioprom	Virin-ensh
Mamestra brassicae	(NPV)	USSR	Glavmikrobioprom	Virin-ex
Neodiprion lecontei	NPV	Canada	Canadian Forestry Service† Sault Ste. Marie	-
Neodiprion sertifer	NPV	USA	Indiana Farm Bur. Co-op Assoc.	Polyvirocide
			U.S. Forest Service, Hamden, CT**	-
		UK	Unit of Invertebrate Virology, Oxford‡	-
		Finland	Kemira Oy Co.	-

(Continued)

TABLE 4 (CONTINUED)

Target insect	Virus	Country of origin	Manufacturer	Trade name
Orgyia pseudotsugata	NPV	USA	U.S. Forest Service, Forestry Sciences Laboratory, Corvallis, OR	TM Biocontrol-1*
Pieris sp.	NPV	USSR	Latvian Agr. Acad.	Virin GKB
Prodenia sp.	NPV	USA	Nutrilite Products International Minerals and Chemical Co.	Biotrol VPO Viron/P
Spodoptera exempta	NPV	UK	Ministry of Overseas Development	
Spodoptera littoralis	NPV	UK	London Unit of Invertebrate Virology, Oxford	-
Spodoptera sp.	NPV	USA	International Minerals and Chemical Co. Nutrilite Products	Viron/S Biotrol VSE
Spodoptera litura	NPV	Japan	Chugoku National Agricultural Experiment Station	-
Trichoplusia sp.	NPV	USA	Nutrilite Products International Minerals and Chemical Co. Rudd Associates Biological Control Suppliers	Biotrol VIN Viron/T *Trichoplusia* virus *Trichoplusia* virus

Source: Aizawa, K. (22), Harper, J.D. (24), and Ignoffo, C.M. (25).
WHO Tech. Rep. Ser. (1973). No. 531
Personal communications.

*EPA registration for use granted.

**Submission to EPA for registration imminent.

+Virus preparations under field use and safety testing; registration and production projected.

†Safety testing at an advanced stage -- submission for registration and production expected soon.

TABLE 5. SOME PROPERTIES OF BACULOVIRUSES FROM SPODOPTERA SPP.

	Dimensions		Proteins		DNA (Form III)		
	Polyhedra	Virus particles	Polyhedra	Virus particles	Centrifugation	Reassociation kinetics	EM
Spodoptera littoralis	1.3 μm (50Å)	320 x 153 nm (E) 281 x 38 nm (N)	29300 Degrades to:* 25000	Eleven 107000 to 11000 NP40:** Two 32000 14000	52.9S 83.7×10^6 (71.7×10^6) 1.7055 g/cc G:C 46 (Tm 53)	62.4×10^6	84.5×10^6
Spodoptera exempta	1.1 μm (52Å)	271 x 107 nm (E) 258 x 42 nm (N)	28400 Degrades to: 25000	Ten 85000 to 10000 NP40: One 39000	54.9S 92.3×10^6 (79.3×10^6) 1.7012 g/cc G:C 42 (Tm 50)	82.5×10^6	79.6×10^6
Spodoptera frugiperda	1.1 μm (50Å)	254 x 119 nm (E) 253 x 41 nm (N)	28200 Degrades to: 22600	Ten 90000 to 10000 NP40: Three 48000 36000 33000	50.3S 73.3×10^6 (62.8×10^6) 1.6982 g/cc G:C 41 (Tm 48)	67.8×10^6	73.8×10^6
	Section (Lattice)	Negative staining E = enveloped N = naked	*Alkaline protease action	**NP40: Action of this detergent removes virus envelope		$T4 = 126 \times 10^6$ (108×10^6) 61.8S	$T7 = 25.1 \times 10^6$

Each virus is very similar morphologically.
Each virus is genetically related to some extent on the basis of nucleotide sequence homology.
Each virus will cross react antigenically though with a specific immunodiffusion line pattern.
The virus nucleocapsid antisera react specifically in immunodiffusion tests and FAB.
Each virus will grow in the Spodoptera frugiperda cell line.

Source: Kelly, D.C. (20), Harrap et al. (21), and Bud, H.M., and Kelly, D.C. (26).

efficacy will now be demonstrated in the field. This is the philosophy advocated and followed in Oxford, and other agencies involved with us as research contractors or seeking our opinion have been given this advice.

If such procedures are not followed, there are real dangers of using these viruses irresponsibly. This can be illustrated with an example from a pest control project in Sabah, Southeast Asia, with which we are familiar and which is well known to us in Oxford through material sent in for diagnosis. I referred to it earlier. This analysis forms the third part of this article -- the irresponsible use of pesticidal viruses.

Oil and coconut palms are liable to attack by the limacodid Darna trima. This insect causes devastating damage to foliage -- up to 2,000 larvae per frond have been recorded and insecticidal control has not always been successful. However, natural epizootics have been observed to reduce the population rapidly. When they occur, dead larvae can be collected, ground up, strained through cheesecloth and the filtrate used as a control spray. This method was used in Sabah using 4 ounces of such insect material per 20 gallons of water and spray applications with mistblowers or knapsack sprayers at 20 gallons per acre. The application method depended on the economic threshold of the pest, e.g., 1-50 larvae per total palm, spray every tenth row; 50-100 every fifth; 100-200 every second row, and so on. Using this method, control was dramatically successful, virtually 100% in 5-6 days with one spray application at a cost only 40% that of chemical pesticides (13).

Our laboratory in Oxford had identified two sizes of small spherical virus in D. trima insects from Sarawak in 1972. Last year material was received from Sabah for diagnosis -- material equivalent to that in use for the control program outlined above. We found three viruses: a granulosis virus (Baculovirus), a 27 nm RNA spherical virus (enterovirus-like) having four structural polypeptides, and a 34 nm RNA spherical virus (calici-like) having one polypeptide.

We do not know which virus was most effective in the pest control program or indeed whether the mortality achieved indicated a combined effect of the three viruses isolated. What this example clearly indicates is that insects can support replication of several viruses, often simultaneously, and without proper purification and identification one simply does not know how the control is being achieved, how to calibrate it, or what the hazards might be. In this instance, two of the viruses isolated have features in common with viruses of vertebrates. Indeed, work by Dr. F.O. MacCallum in our laboratory has shown positive antigen-antibody reactions with one or other of the two RNA spherical viruses in human sera from Malaysia. Previous work has indicated positive antigen-antibody reaction with such viruses and sera of several species of wild and domestic animals in which antibody has presumably been elicited with a related virus to which the animal had been exposed.

This example, then, shows both the dramatic success possible using a virus to control a pest insect and underlines the dangers which might be inherent in the "bug juice" do-it-yourself approach -- a method that has even been advocated in the U.S. by well-meaning publications. The argument of a "safe," "natural" method of pest control which finds favor with ecologists and conservationists might well hold other dangers for our environment. What is more -- and relevant to those of us at this conference -- disillusion with the rate of progress in developing conventional biological control methods could encourage the do-it-yourself approach, thereby creating the very hazards we are trying to avoid.

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DISCUSSION

LONGWORTH: I would like to make a comment regarding the Darna trima story. None of us would condone such an approach, but isn't it true, Dr. Harrap, that there were no fatalities that we know of, in fact, there were far, far less than was the case with the conventional insecticides that had been used before?

HARRAP: That is true. There have been no reports of adverse clinical symptoms or pathologies in the plantation workers. The only evidence we have of anything possibly occurring, and this does not come from the same plantation, is the antigen-antibody reactions. In fact, there were more problems on that plantation relative to reactions to defoliants and chemical pesticides than to viruses. This does not, of course, mean that something more insidious or more subtle might be occurring.

ZAITLIN: Of course, the ultimate recipients of these sprays are plants. In your testing program, do you test the effect of viral pesticides on plant species?

HARRAP: We are not set up for such tests on plants in our laboratory.

STOLLAR: Could you clarify the terms "baculovirus" and "nuclear polyhedrosis"?

HARRAP: The preferred term suggested by the International Committee is "baculovirus." This term covers both granulosis and nuclear polyhedrosis viruses. The difference is that the nuclear polyhedrosis virus has a large polyhedron-like inclusion body with many virus particles located in it. The granulosis virus has a granule, capsule-like inclusion body with one virus particle in it. To all other intents and purposes, they are very similar and have been combined as baculoviruses.

PART II
VIRUSES: BIOLOGY AND IDENTIFICATION

Baculoviruses

Max D. Summers, Ph.D.
Texas A&M University
College Station, Texas

INTRODUCTION

Nuclear polyhedrosis (NPV) and granulosis viruses (GV) contain DNA genomes, are classified in the family Baculoviridae (1), and are presently identified on the basis of morphology and host specificity (2). The characteristic structure associated with the virus is a proteinic inclusion ranging in size from 1-15 microns in which rod-shaped enveloped nucleocapsids are randomly embedded. The NPVs exhibit two major structural relationships: inclusions which contain enveloped single nucleocapsids, or those containing multiples of nucleocapsids common to a single envelope (3). All granulosis viruses are similar in structure: the typical inclusion body is ovicylindrical with sizes from different species of approximately 250-300 x 450-550 nm, and they usually contain only one virus per inclusion. The structure of the enveloped nucleocapsid, as observed by electron microscopy, is similar to that of the NPVs; an enveloped rod-shaped particle with dimensions of 40-60 nm x 350-400 nm.

Initially, baculoviruses were considered specific for insect arthropod hosts. The discovery of the pink shrimp Penaeus duorarum NPV (4, 5) has altered this concept. Nonoccluded particles with baculovirus structure have been observed in nuclei of hemocytes and connective tissue elements of the European crab, Carcinus maneas (6), and in the epithelium of hepatopancreatic cells of the blue crab, Callinectis sapidus (7). Observations of this nature

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led to curiosity and speculation about biological and structural relatedness of these arthropod viruses. This will ultimately require comparisons of insect baculoviruses with structurally similar viruses of noninsect invertebrates.

Polyhedron Structure

Biochemical studies on the crystal inclusion of NPVs and GVs have identified as the major component only one polypeptide (3, 8-14). After alkali solubilization and purification, this protein is referred to as granulin for GVs and polyhedrin for NPVs (12).

The crystal structure dissociates under dilute alkaline saline conditions to give components sedimenting at approximately 12S (9, 12, 15-17). Dependent upon the virus studied, molecular weights for the subunit based upon sedimentation properties are estimated to range from 180,000 to 378,000 (Table 1). For some purified granulins and polyhedrins, the isolated subunits are apparently capable of aggregation into higher order polymers (9, 13, 18). Treatment of the solubilized granulin or polyhedrin with SDS and electrophoresis in SDS-polyacrylamide gels shows that the basic unit of structure is a smaller molecule which can vary slightly in size with species (3, 14, 17, 19, 20). Using vertical slab polyacrylamide gel electrophoresis, mobility differences can be shown for granulins and polyhedrins ranging from 25,500 to 31,000 daltons (Figure 1, Table 1).

Various protein denaturants and solubilization procedures in addition to alkali have been successfully employed to dissociate the crystal of *Trichoplusia ni* GV at neutrality (21) and the activity of the denaturing agents on crystal structure correlated with ultrastructural alterations (22). Each denaturating agent produced characteristic alterations in crystal structure; however, enveloped nucleocapsids were only obtained by carbonate (weak alkali) solubilization. Ultrastructural comparisons of crystal dissolution in the midgut lumen of *Trichoplusia ni* larvae showed that carbonate solubilization *in vitro* approximated that observed to occur *in vivo*.

Harrap et al. (25) studied the serological and structural properties of *Spodoptera littoralis*, *Spodoptera exempta*, and *Spodoptera frugiperda* NPVs. All three viruses were morphologically similar (MNPVs) and by electron microscopy indistinguishable from each other. As compared by SDS-polyacrylamide gel electrophoresis there was no significant difference in size among polyhedrins. There were two bands; a major band of 28-29,000 and a smaller band

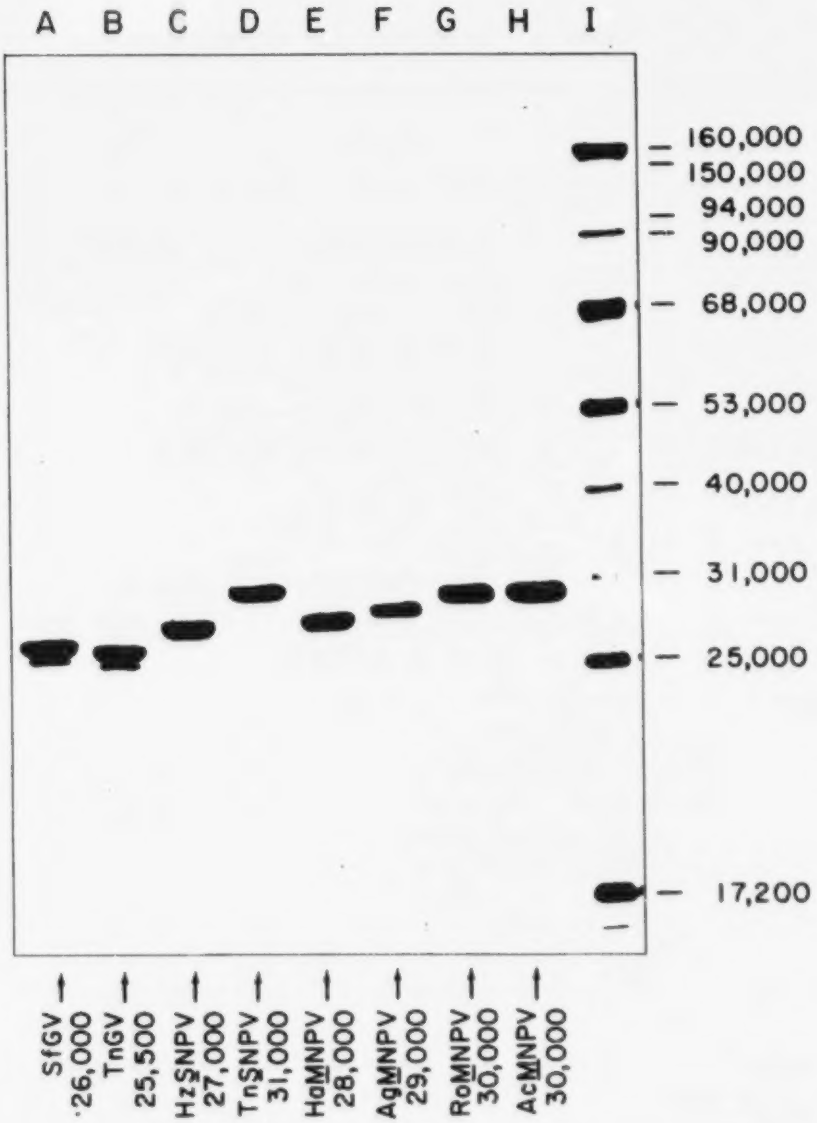
A
B
C
D
E
F
G
H
I
160,000
150,000
94,000
90,000
68,000
53,000
40,000
31,000
25,000
17,200
SfGV 26,000
TnGV 25,500
HzSNPV 27,000
TnSNPV 31,000
HaMNPV 28,000
AgMNPV 29,000
RoMNPV 30,000
AcMNPV 30,000

Figure 1. Molecular weights and relative mobilities of granulins and polyhedrins disrupted in 2% SDS and 5% mercaptoethanol. 1.5 μg of each was electrophoresed in 11% polyacrylamide slab gels in the presence of 0.1% SDS. SfGV and TnGV granulin, and HzSNPV, TnSNPV, HaMNPV, AgMNPV, RoMNPV, and AcMNPV polyhedrin are in A through H, respectively. The molecular weights of the standard protein markers are: RNA polymerase subunits, 160,000, 150,000, 90,000 and 40,000; phosphorylase-a, 94,000; bovine serum albumin, 68,000; L-glutamic dehydrogenase, 53,000; DNA nuclease, 31,000; chymotrypsinogen, 25,000; and myoglobin, 17,200. Relative migrations are indicated adjacent to the virus polypeptides.

TABLE 1. GRANULINS AND POLYHEDRINS

Virus	$s_{20,w}$	MW*†	References
B. mori SNPV	6.26 *S* (pH 11)	120,000*	(23)
	13.6 *S* 19.7, *S*		
	29.7 *S* 38.0 *S* (pH 7)		(23)
	8.76 *S*, 3.35 *S* (pH 8.5)		(23)
T. ni SNPV	11.0 *S* (pH 11)		(24)
P. dispar MNPV	11.3 *S*, 11.5 *S* (pH 11)		(16)
P. brassicae GV	12.5 *S*, 16.0 *S*		(9)
	2 to 4 *S* (pH 11)		
T. ni GV	7.25 (pH 11)	180,000*	(12)
	3.45 *S* (minor)		
	15.8 *S* (minor)		
T. ni SNPV		31,000†	(20)
AcMNPV		30,000†	(20)
RoMNPV		30,000†	(12)
AgMNPV		29,000†	(12)
HaMNPV		28,000†	(12)
HzSNPV		27,000†	(12)
TnGV		25,000†	(13)
SfGV		26,000†	(13)
PdSNPV		50,000†	(12)
B. mori SNPV	2.0 S	28,000†	(17)
G. mellonella NPV	2.0 S	28,000†	(17)
S. frugiperda NPV		28-29,000†	(25)
S. exempta NPV		28-29,000†	(25)
S. littoralis NPV		28-29,000†	(25)
O. pseudotsugata SNPV	12 S	26,000†	(18)
O. pseudotsugata MNPV	12 S	26,000†	(18)
Porthetria dispar NPV		30,350	(26)
Prothetria dispar NPV		29,000	(27)

*Sedimentation.

†Polyacrylamide gel electrophoresis in the presence of 10% SDS.

suspected to be a product derived by proteolytic cleavage. Production of the lower molecular weight component was time dependent and could be inhibited by heat and excessively high pH.

During alkali-solubilization of polyhedra purified from infected insects a protease can be activated (3, 13). Although the protease appears to be present in occluded virus extracted from infected insects, conformation of similar activity from occluded virus purified from cell culture is yet to be established. If the enzyme is not inactivated by the appropriate inhibitor or heat treatment procedure, hydrolytic activity can produce several lower molecular weight products.

Comparisons of the primary structure of purified granulins and polyhedrins (undegraded) has been conducted by two dimensional high voltage electrophoresis which can discriminate single amino acid differences among major peptides (14). All polyhedrins and granulins studied [*Trichoplusia ni* SNPV (TnSNPV); *Rachiplusia ou* MNPV (RoMNPV); *Autographa californica* MNPV (AcMNPV); *Trichoplusia ni* GV (TnGV); and *Spodoptera frugiperda* (SfGV)] had similar primary structures; yet each virus had different peptides, indicating regions of nonsimilarity. TnGV and SfGV granulins showed the closest degree of similarity as compared to polyhedrins. Each NPV or GV studied to date by peptide mapping has shown distinct polyhedrin or granulin structure (28, 29). The evidence indicates that these are virus specified but group related proteins. The primary sequence for *Bombyx mori* polyhedrin has been determined (11).

The most thoroughly characterized granulin or TnGV has chemical and physical properties which should be investigated and confirmed as properties consistent with other granulins and polyhedrins. The acidic protein is apparently phosphorylated, phenol soluble, and contains a high concentration of hydrophobic amino acids. Phosphorylated proteins are routinely identified as structural components of RNA and DNA vertebrate viruses, although the determination of a biological function for phosphorylated proteins has been difficult. Certain reports suggest a regulatory relationship with a viral kinase and/or possible relationships to regulatory processes involving transcriptase activity (30). A class of phenol soluble, nuclear acidic proteins in eukaryotic systems has been the object of investigation relative to possible regulatory role(s) during growth and differentiation (31).

Virus Structure

By use of the standard technique (adding dilute alkaline saline to purified polyhedra at a pH of 10.9) the enveloped nucleocapsid can be released and purified (16, 32-36). In a GV of SNPV, the enveloped nucleocapsid will usually occur as a single band upon sedimentation or isopycnic centrifugation. If it is of the multiple type (MNPV), several bands will be observed in the gradient after rate zonal, or centrifugation to equilibrium. In CsCl each band will differ in density according to the number of nucleocapsids per envelope with densities ranging from approximately 1.20-1.25 g/ml to 1.32 g/ml (20, 25, 36).

Studies have shown that the qualitative and quantitative nature of multiple nucleocapsid banding profiles may be strain or species specific. Each of the three *Spodoptera* NPVs reported by Harrap et al. (25) had a characteristic banding profile after centrifugation to quasi-equilibrium in sucrose gradients. *S. exempta* consistently showed a small number of virus bands, five as compared to *S. frugiperda* and *S. littoralis*; each of the latter had eight. There was no similarity or matching in position or quantity in each of the enveloped nucleocapsid peaks among the viruses studied.

Removal of the envelope by the appropriate detergent treatment produces a nucleocapsid with a characteristic structure (8, 37-39). The nucleocapsid has a density of about 1.48 g/ml after isopycnic banding sucrose or in CsCl gradients (20, 25). Electron microscope observations suggest that one end structure of the nucleocapsid is involved in a nuclear pore interaction to uncoat the DNA genome early during the infection process (40, 41).

Virus Structural Polypeptides

For serological studies it is important to establish and utilize reproducible criteria for virus or antigen purity (20, 32, 42). Polypeptide profiles derived from SDS-polyacrylamide gel electrophoresis (SDS-PAGE) are one such criterion.

Baculovirus enveloped nucleocapsids have several structural proteins ranging in size from 8,000 to more than 100,000. Using SDS-PAGE comparison of polypeptides for NPVs and GVs shows that each virus has, qualitatively and quantitatively, a different polypeptide composition. SDS-PAGE of the NPV structural polypeptides of *Porthetria dispar* (PdMNPV) (27) and *Trichoplusia ni* NPV (43) shows the presence of 14 and 12 major polypeptides, respectively. A preliminary report by McCarthy and Liu (26) shows 25 proteins present in the PdMNPV enveloped nucleocapsid. Eleven bands were observed present in enveloped nucleocapsids of *S. exempta* (25). After NP-40 treatment and envelope removal, two major proteins of *S. littoralis* (L32 and L14) were observed to be nucleocapsid proteins, and E39 for *S. exempta* and F36 and F33 for *S. frugiperda*.

Summers and Smith (20) studied the relative mobilities of SDS-treated structural polypeptides of eight baculoviruses (Figure 2). Depending on the baculovirus, a range of 15-25 bands was resolved. Some bands exhibited similar mobility for different viruses, for example AcMNPV and RoMNPV appeared closely related; however, each virus had a distinct composition when comparing relative mobility and the number of bands.

After centrifugation to quasi-equilibrium in sucrose gradients, the least dense band (1.20-1.21 g/ml) of enveloped single nucleocapsids from AcMNPV, RoMNPV, *Anticarsa gemmitalis* (AgMNPV) and *Heliothis armigera* (HaMNPV) were separated from the multiples (usually 7-8 additional bands ranging in density from 1.22 to 1.25 g/ml), and compared by SDS-PAGE (Figures 3-5). Qualitative and quantitative differences exist between multiples and singles for each virus. For example, VP30, VP28, VP19, VP18.5, and VP16 of AcMNPV singles were observed to be in relatively lesser concentration when compared to the same bands in multiples. VP58, VP36, and VP23 are apparently absent or diminished in relative concentration to a level not detectable in the gels (Figure 5). The polypeptide composition of HaMNPV singles shows that VP64, VP47, VP28, and VP20 are present in increased concentration relative to the same protein in multiples, VP89, VP75, VP62, and VP51 were not observed in multiples. In contrast, there is more of HaMNPV VP45 in multiples as compared to singles. Similar relationships were observed for the other MNPVs as well (Figure 3).

The envelopes of AcMNPV, RoMNPV, and TnGV were removed by treatment with NP-40. The other five viruses were resistant to this chemical treatment and yielded mostly intact virus (20). The buoyant densities of AcMNPV, RoMNPV, and TnGV nucleocapsids and capsids, after banding in cesium chloride gradients, were approximately 1.48 g/ml and 1.33 g/ml, respectively. AcMNPV capsids were composed of two major peptides, VP37 and VP18.5, and trace quantities of VP30.5 and VP30 (Figure 6). RoMNPV capsids had several major polypeptides; VP16, VP17, VP18, VP30, and VP36, with detectable quantities of VP30.5. The TnGV capsid was composed of two major polypeptides VP31 and VP17, with trace quantities of VP29 and VP26. Each capsid preparation consistently contained several other minor polypeptides which could be components

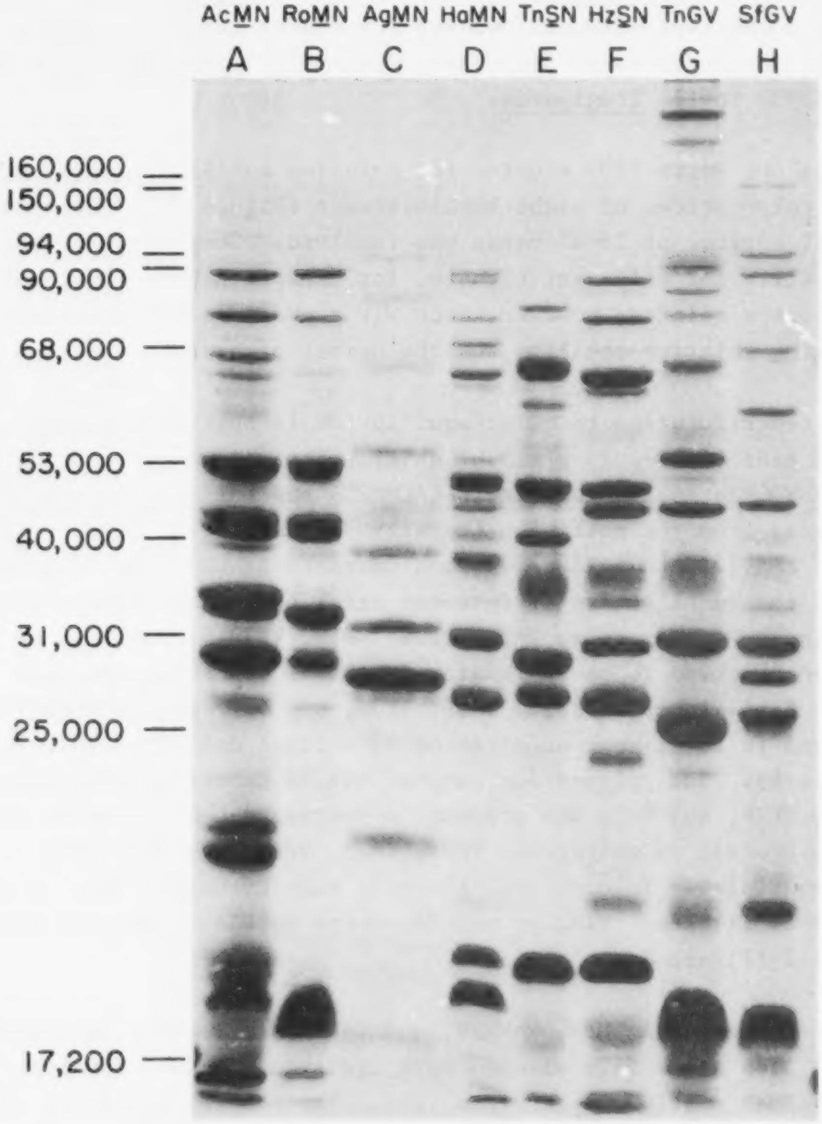
AcMN
RoMN
AgMN
HaMN
TnSN
HzSN
TnGV
SfGV
A
B
C
D
E
F
G
H
160,000
150,000
94,000
90,000
68,000
53,000
40,000
31,000
25,000
17,200

Figure 2. Polyacrylamide slab gel electrophoresis of enveloped nucleocapsid polypeptides. The viruses were purified by equilibrium banding in sucrose (1.17 to 1.25 g/ml) after being liberated from the polyhedra with 0.1 M Na_2CO_3, 0.01 M EDTA, 0.17 M NaCl at 0° to 4°C for 60 min. The band containing only one nucleocapsid per envelope (singles) was removed from the gradient, prepared for electrophoresis by disruption in 2.0% SDS and 5.0% mercaptoethanol for 3 min at 100°C at a protein concentration of 1 mg/ml; a total of 25 μg protein was electrophoresed in 11% polyacrylamide. The virus polypeptides (VP) of AcMNPV, RoMNPV, AgMNPV, HaMNPV, TsSNPV, HzSNPV, TnGV, and SfGV are shown in A, B, C, D, E, F, G, and H, respectively.

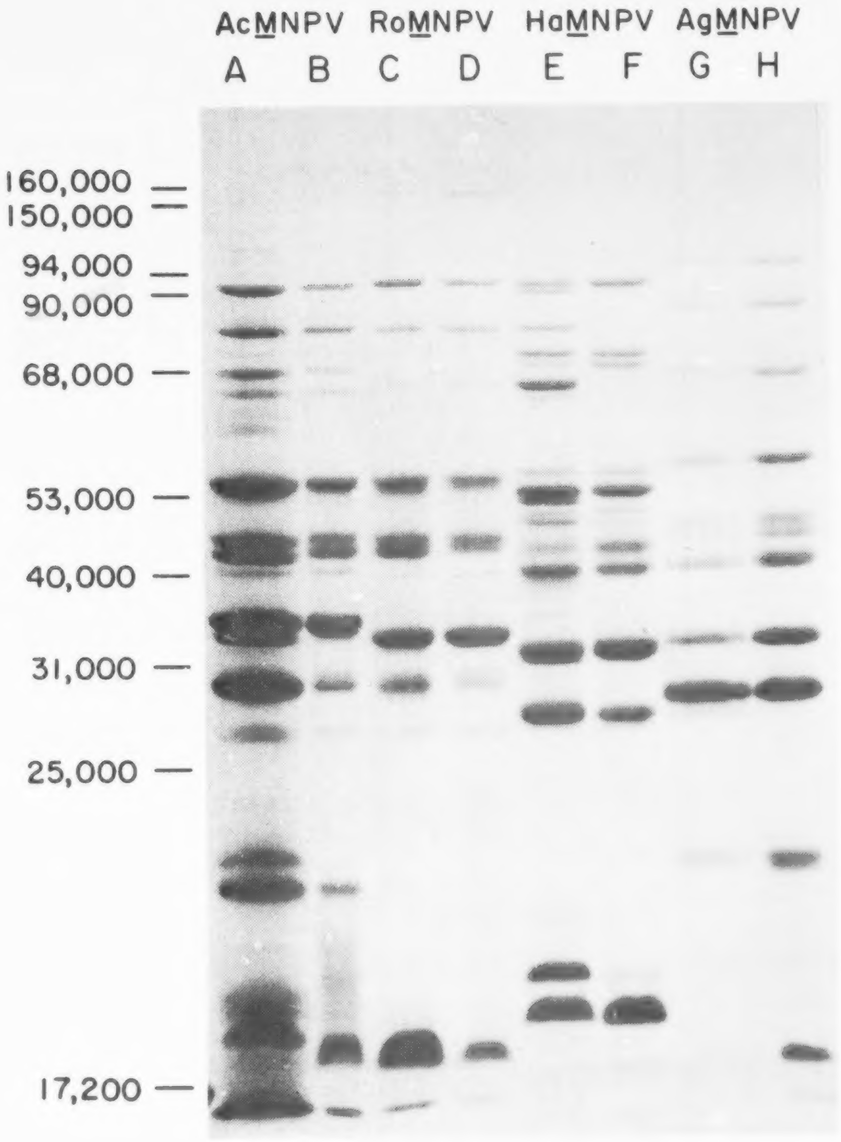
AcMNPV
RoMNPV
HaMNPV
AgMNPV
A
B
C
D
E
F
G
H
160,000
150,000
94,000
90,000
68,000
53,000
40,000
31,000
25,000
17,200

Figure 3. Comparison on a 11% polyacrylamide slab gel of polypeptides from enveloped single nucleocapsids and multiples of nucleocapsids. Each of the four MNPV viruses used in this study was liberated from the polyhedra and gel electrophoresis conducted as described in Figure 2; except for collecting the top virus band (singles) from the sucrose gradient, the rest of the virus bands (multiples) were collected and analyzed. The VPs of singles and multiples, respectively, of AcMNPV are observed in A and B, RoMNPV in C and D, HaMNPV in E and F, and AgMNPV in G and H. The positions of the molecular weight standards are indicated next to the VPs.

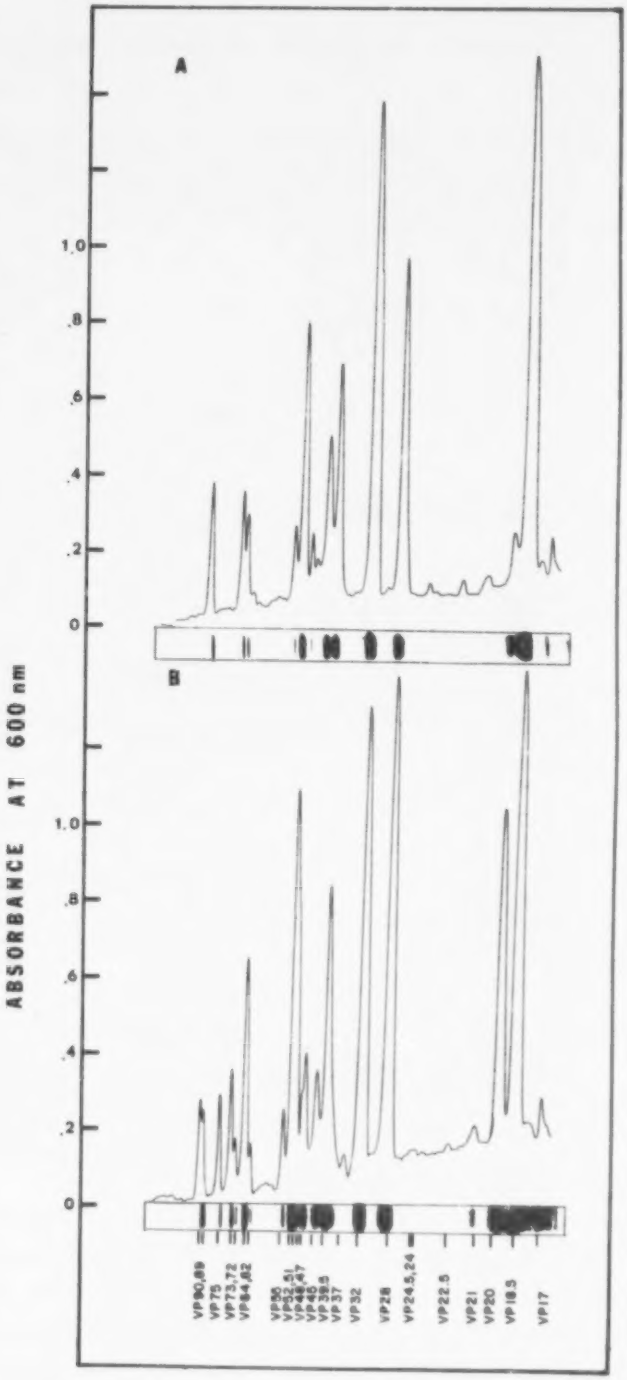

Figure 4. Densitometer tracings (600 nm) from transparencies of the stained envelope nucleocapsid polypeptides from (A) HaMNPV bundles and (B) HaMNPV singles that were electrophoresed as described in Figure 2.

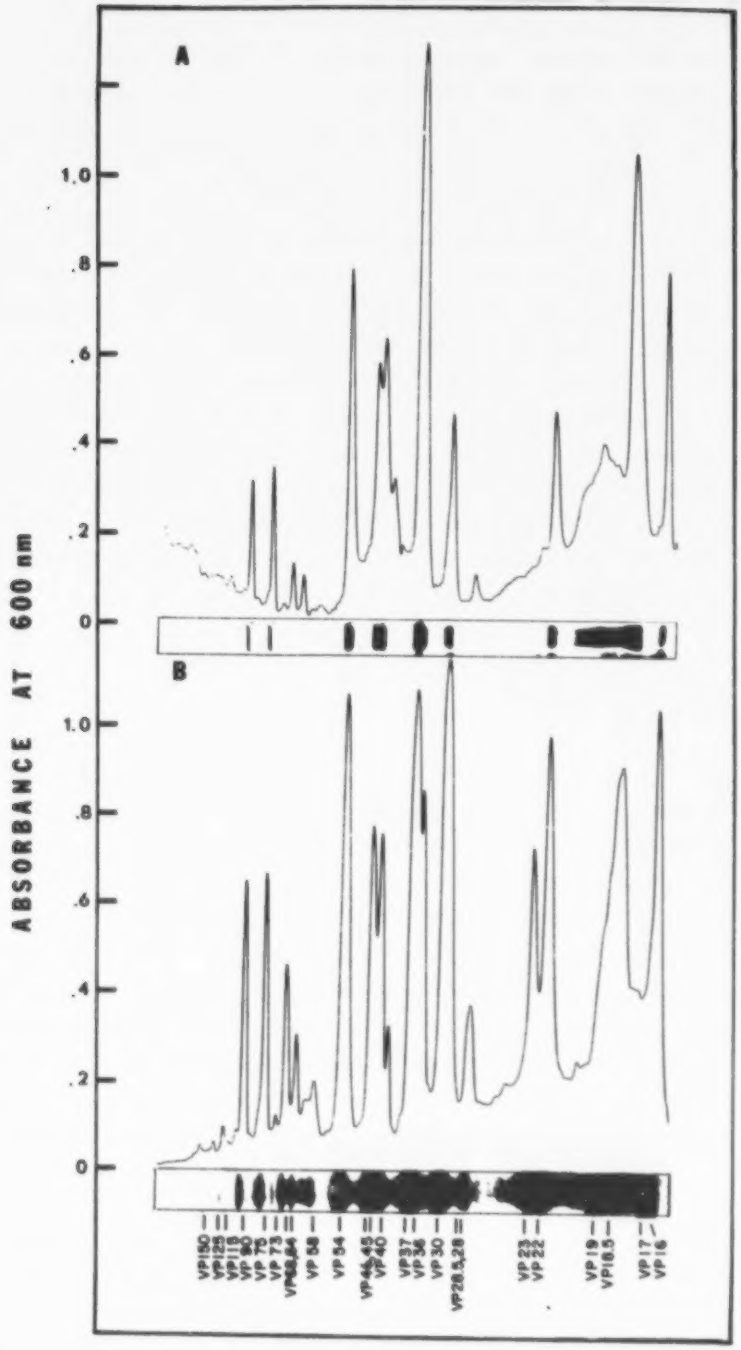

Figure 5. Densitometer tracing (600 nm) from transparencies of stained enveloped nucleocapsid polypeptides of (A) AcMNPV bundles and (B) AcMNPV singles that were electrophoresed as described in Figure 2.

of the viral capsid; however, further study will be needed to identify those proteins. The differences between capsid and enveloped nucleocapsids shown in Figure 6 suggest that the envelope composition of baculoviruses is complex (25). For example, AcMNPV contains approximately 18 bands of which only 3 are capsid proteins.

Payne (44) isolated nucleocapsids from the Oryctes baculovirus using NP-40. The Oryctes baculovirus has certain structural features similar to baculoviruses of Lepidoptera and Hymenoptera. The DNAs are of comparable size and conformation, and serological cross-reactions have been shown to occur with Spodoptera NPVs. However, the Oryctes baculovirus possesses a tail-like structure which is not similar to that of other insect baculoviruses. This structure is similar to that observed on parasitoid particles (40) which have been observed to interact with the nuclear pore of infected cells during the uncoating process. Twelve structural proteins were resolved in the Oryctes baculovirus enveloped nucleocapsid. After treatment with NP-40 eight appeared to be components of the nucleocapsid.

Extracellular or Nonoccluded Virus

The nonoccluded or extracellular virus has different biological and physical properties when compared to those of enveloped nucleocapsids which are occluded and require alkali treatment for liberation from the crystal. During cell infection, at least three and possibly four infectious forms of virus can be present (45): one is the occluded virus which obtains an envelope from probable de novo synthesis in the nucleus and then becomes occluded (46-48); particles which bud from the inner nuclear membrane into the cytoplasm (46, 47, 49); and nucleocapsids which bud through from plasma membrane (36, 46, 47, 49) into the cell culture medium or insect blood. The occluded, the budded, and the intracellular viruses are all reported as infectious, although it has not been verified whether the nucleocapsid is an intracellular infectious form.

A number of studies have reported on the properties of the nonoccluded infectious forms occurring in hemolymph and cell culture medium (36, 50-55). However, in most in vitro studies the extracellular virus was collected from the cell culture medium after extensive cell lysis. It is probable that the intracellular enveloped, unenveloped, and extracellular infectious forms were all included under the somewhat broad classification of nonoccluded virus (47, 51). Therefore, confusion existed as to the nature and identity of the nonoccluded infectious forms.

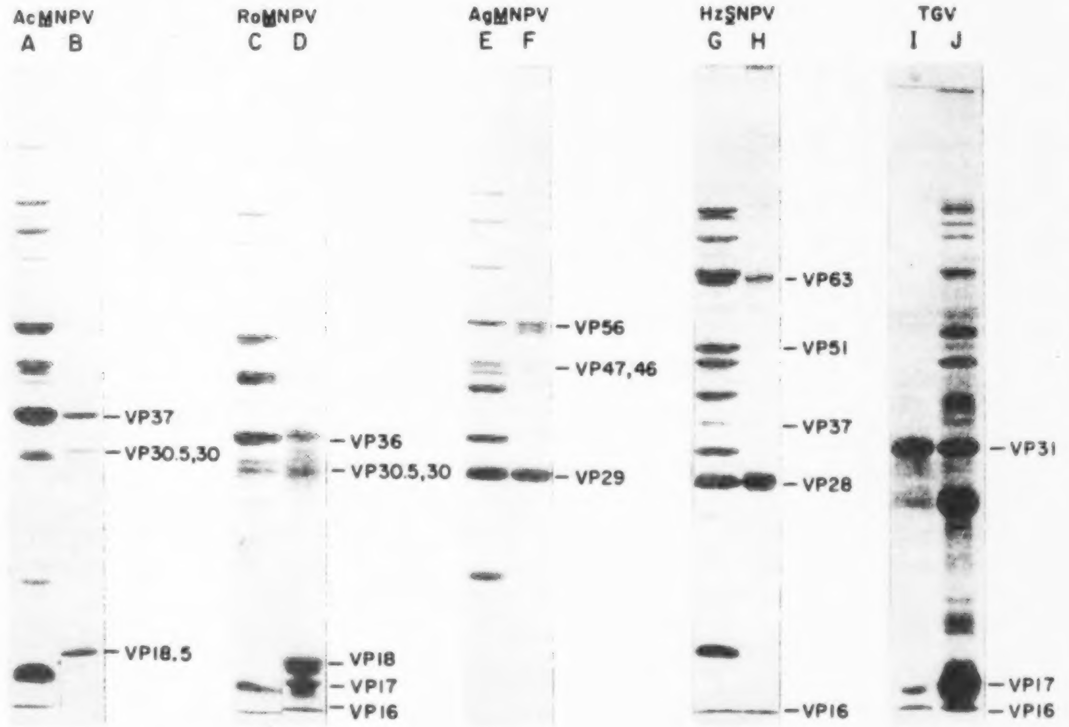
AcMNPV
A B
RoMNPV
C D
AgMNPV
E F
HzSNPV
G H
TGV
I J
VP37
VP30.5,30
VP18.5
VP36
VP30.5,30
VP18
VP17
VP16
VP56
VP47,46
VP29
VP63
VP51
VP37
VP28
VP16
VP31
VP17
VP16

Figure 6. AcMNPV, RoMNPV, and TnGV enveloped nucleocapsids were treated with 2% NP-40 in 0.01 M Tris (pH 7.8), 0.01 M EDTA for 18 hr at 37°C, virus capsids (density of 1.33 g/ml) recovered, and 5 μg of each prepared for electrophoresis in 11% polyacrylamide slab gels. AcMNPV, RoMNPV, and TnGV capsid proteins are shown in B, D, and I, respectively. AgMNPV enveloped nucleocapsids (F) were treated with 2% NP-40 in 0.01 M Tris (pH 7.8), 0.01 M EDTA, 1 M NaCl for 18 hr at 37°C, then centrifuged and electrophoresed. HzSNPV enveloped nucleocapsids (H) were treated with 2% NP-40, 0.01 M Tris (pH 7.8), 0.01 M EDTA, 1 M NaCl for 18 hr at 37°C, then given an additional treatment of 1% mercaptoethanol and incubation for 1 hr at 70°C. The capsid bands were removed from CsCl gradients and 5 μg of each was electrophoresed as described previously. Adjacent to each capsid preparation is a nontreated control of enveloped nucleocapsids that had been repurified on CsCl gradients.

Summers and Volkman (36) compared the properties of culture-derived, plasma membrane-budded nonoccluded virus (PMB-NOV) from RoMNPV and AcMNPV to those of alkali-liberated enveloped nucleocapsids. If collected at 24 h PMB-NOV were loosely enveloped single nucleocapsids with a distinct surface modification. Infectious virions obtained from insect hemolymph had physical and biological properties similar to particles obtained from culture medium. Both the PMB-NOV and the virus obtained from hemolymph banded at a density of 1.17-1.18 g/ml. The alkali-released virions were for the most part multiple nucleocapsids which banded at various densities. The buoyant density of each depended on the number of nucleocapsids per envelope and ranged from 1.20 g/ml for enveloped single nucleocapsids to 1.25 g/ml for multiples. The envelopes from alkali-liberated virus were relatively tight fitting with the nucleocapsid and were devoid of surface projections.

Volkman et al. (45) showed that alkali-liberated and nonoccluded forms were antigenically different by neutralization studies. Antisera raised against purified AcMNPV alkali-liberated virus (which we described as LOVAL: larval occluded virus alkali-liberated) did not neutralize the AcMNPV nonoccluded virus from infectious hemolymph, alkali-treated and untreated NOV from TN-368-10 cells or infectious virus liberated from fat body cells. It did neutralize both AcMNPV and RoMNPV LOVAL. Antiserum to RoMNPV LOVAL did not neutralize RoMNPV NOV but did neutralize both RoMNPV and AcMNPV LOVAL. Antisera raised against AcMNPV PMB-NOV neutralized not only all nonoccluded forms of both AcMNPV and RoMNPV tested, but the alkali-liberated infectious virus forms as well. Absorption of the PMB-NOV antisera with AcMNPV and RoMNPV LOVAL removed all of the anti-LOVAL neutralization activity, but did not diminish anti-NOV neutralization titers. This shows that whereas the alkali-liberated and nonoccluded forms of the virus share some antigenic determinants, those involved in the neutralization of the two different infectious forms are different.

Physical-infectious particle calculations showed that PMB-NOV AcMNPV from the culture medium of TN-368-13 cells is 1,700-fold more infectious than LOVAL multiples and 1,900-fold more infectious than LOVAL singles as titrated *in vitro* (Table 2).

TABLE 2. AcMNPV PHYSICAL-INFECTIOUS PARTICLE RATIOS AS ASSAYED IN VITRO (45)

Virus prepn	DNA μg/μg of protein	DNA g/μg of protein (x 10^{-7})	Particle/ μg of protein (x 10^{9})	PFU/μg of protein	Particles/ PFU
PMB-NOV	0.19	1.9	1.1	8.9 x 10^{6}	1.28 x 10^{2}
LOVAL I*	0.20	2.0	1.2	5.0 x 10^{3}	2.4 x 10^{5}
LOVAL II-X†	0.30	3.0	1.8	8.4 x 10^{3}	2.2 x 10^{5}

*Single nucleocapsid per envelope

†Many nucleocapsids per envelope

Baculovirus DNAs

Baculoviruses contain double-stranded DNA of high molecular weight which is covalently closed (56). The properties of a few baculovirus DNAs have been investigated by comparing extraction procedures, sedimentation, electron microscopy, and renaturation kinetics.

Summers and Anderson (57-59) studied GV and NPV DNAs using sedimentation relative to that of bacteriophage T4 DNA. The molecular weight for *Trichoplusia ni* GV was estimated at 99-119 x 10^{6}; *Spodoptera frugiperda* GV, 95-114 x 10^{6}; *Trichoplusia ni* SNPV 99-119 x 10^{6}; *Rachiplusia ou* MNPV, 95-114 x 10^{6}; *Spodoptera frugiperda* MNPV, 95-114 x 10^{6}. Sedimentation coefficients were 60*S*, 59*S*, 60*S*, 59*S*, and 59*S*, respectively.

Centrifugation to equilibrium in ethidium bromide-CsCl gradients revealed the presence of two bands of DNA at 1.54 g/ml and 1.58 g/ml which were double-stranded linear, and relaxed circular DNA (light band), and covalently closed, double-stranded DNA (heavy band). The covalently closed and relaxed circular DNA forms co-sedimented in sucrose gradients with an ionic strength greater than 10^{-3}. By using gradients with the appropriate ionic strength, three sedimenting forms could be detected: super-coiled DNA (ccDNA or Form I), relaxed circular DNA (rcDNA or Form II), and double-stranded, linear DNA (dlDNA or Form III). Using the classical technique of nicking with low concentrations of pancreatic deoxyribonuclease, a discontinuous conversion of circular (Form II) to double-stranded linear DNA (Form III) occurred, and a continuous conversion from double-stranded linear to lower molecular weight fragments of random sizes.

Summers and Anderson (57) observed that lyophilization of occluded virus preparations resulted in the inability to recover appreciable quantities of covalently closed DNA. This suggested that the lyophilization process induced a single strand sisson into the phosphodiester linkage. The reasons for this were not clear; however, it was suspected that an endonuclease may have been responsible.

It was noted that ccDNA sedimented approximately 3 to 4 times faster in alkaline sucrose gradients as compared to denatured linear and circular DNAs; this was behavior typical of denatured, covalently closed DNA. Co-sedimentation of GV and NPV DNAs showed that TnGV and SfGV were slightly different, although denatured GV DNAs sedimented faster than the NPV DNAs. Denatured NPV DNAs could not be differentiated from each other by sedimentation in alkaline sucrose gradients compared by relative sedimentation with denatured T4 DNA. Denatured TnGV and SfGV DNAs had sedimentation coefficients of 59S and estimated molecular weights of 84×10^6, a value which is closer to more accurate measurements on the sizes of NPV DNAs (60).

Harrap et al. (25) studied the chemical and physical properties of three Spodoptera NPVs. Two bands of labeled DNA were obtained after banding in ethidium-bromide-CsCl gradients separated by a density of 0.04 g/ml. On neutral sucrose gradients the viral DNA preparations sedimented as three bands: the fastest (68.4S) was ccDNA; the major sedimenting band at 57.5 to 61.8S was rcDNA; and the 50.3 to 54.9S band was dlDNA. Molecular weight estimates for the three NPV DNAs were: S. littoralis, 71.7 to 83.7×10^6; S. exempta, 79.8 to 92.3×10^6; and S. frugiperda, 62.8 to 73.3×10^6. Differences in sedimentation between S. littoralis and S. exempta could not be detected by co-sedimentation. All three DNAs had different densities of 1.7056, 1.7010, and 1.7005 g/ml for S. littoralis, S. exempta, and S. frugiperda, respectively. Comparison of thermal denaturation profiles showed that values for GC content calculated by melting profiles and those derived from densities did not agree closely; for example, 46.5% GC estimated from density, and 52-53% G+C determined by T_m for S. littoralis DNA.

Kelly (61) studied the DNAs of four closely related Spodoptera NPVs (Spodoptera frugiperda, S. exempta, S. exigua, and S. littoralis) assessing sizes and degree of homologies by DNA reassociation kinetics. Molecular weights (relative to bacteriophage T4, 126 x 10^6) determined by reassociation kinetics were 66.8 x 10^6, 82.5 x 10^6, 65.4 x 10^6, and 62.4 x 10^6, respectively. The extent of sequence homology between viral DNAs was determined by allowing ^{125}I-labeled DNA fragments to reassociate in the presence of a vast excess of homologous or heterologous DNA. The data revealed that the four viruses were genetically related to some degree. _S. exigua_ and _S. exempta_ genomes were more closely related (approximately 60%) than the genome of _S. frugiperda_ and _S. littoralis_ (approximately 15%).

Two NPVs (MNPV and SNPV) have been isolated from the Douglas fir tussock moth, _Orgyia pseudotsugata_ (62). The DNAs were analyzed by reassociation kinetics using both optical renaturation and S_1 nuclease assay. Genome size was also analyzed by analytical ultracentrifugation, and base composition by melting properties.

The OpMNPV had a T_m of 76.9 and an estimated G+C content of 54%. Renaturation kinetics using optical methods and _Escherichia coli_ (_E. coli_) DNA as a standard gave an estimated genome size of 86 x 10^6. This calculation was made after correction for G+C content. Reassociation using ^{125}I-labeled DNA was examined and compared with lambda phage and _E. coli_ DNAs with the extent of annealing determined by digestion with S_1 nuclease. DNA reassociated at a rate of an equivalent genome size of 70-80 x 10^6 daltons. Reassociation of the OpMNPV DNA followed second order reaction kinetics. The data indicated that the viral DNA is composed mainly of unique sequences although the presence of any small repeated portion could not be excluded.

DNA size was also determined by sedimentation. The DNA preparation analyzed by equilibrium banding in an ethidium bromide-CsCl gradient was shown to be in the linear form. However, documentation of the presence or absence of circular molecules was not made using rate-zonal sedimentation. The estimate derived by sedimentation for each NPV DNA was 96 x 10^6 daltons.

Most molecular weights of baculovirus DNAs have been derived by sedimentation in neutral sucrose gradients relative to bacteriophage DNA standards and/or renaturation kinetics. Better confirmation of size using more accurate means of measurement has been provided by Burgess (60). Molecular weight estimates were made on ccDNAs extracted from seven NPVs and three GVs. By a comparison of contour length with that of the replicative form of Fl bacteriophage DNA, the molecular weights ranged from 58 x 10^6 to 94 x 10^6 for ten baculoviruses: _Spodoptera frugiperda_, 80 x 10^6; _Spodoptera littoralis_, 80 x 10^6; *Epiphyas postvittana*, 58 x 10^6; *Pseudaletia separata*, 94 x 10^6; *Heliothis armigera*, 77 x 10^6; *Chrysodeixis eriosoma*, 77 x 10^6; *Laspeyresia pomonella*, 71 x 10^6; and *Wiseana cervinata* GV, 69 x 10^6.

Skuratovskaya et al. (63) studied the size and infectivity of supercoiled DNA purified from the NPV of *Galleria mellonella*. Separation of covalently closed DNA from linear and open molecules (contour lengths 50-52 microns, 96-100 x 10^6) was achieved by centrifugation to equilibrium in ethidium bromide-CsCl. Both bands were infectious as tested by injection.

Scharnhorst et al. (64) observed DNA molecules from *Heliothis zea* SNPV by the spreading technique and detected a heterogenous population of molecules with contour lengths ranging from 15-45 microns. The most prominent sizes observed were of approximately 20-25 microns. Thirty-six percent of the molecules existed as a supercoiled form. An internal standard was not utilized for a direct comparison and measurement. The molecules with lengths of 20-25 microns had molecular weights of 40-50 x 10^6. This corresponded to their unpublished estimates derived from DNA reassociation kinetics.

Baculovirus Infection and Maturation

A comparison of cellular infection *in vivo* (insects) with that shown to occur *in vitro* (cell cultures) reveals that some aspects of the infection process are similar, yet there are important differences. In nature the primary infection is accomplished by the occluded form (polyhedra) of the virus. However, secondary infection occurs by the nonoccluded or extracellular form of the virus, a process analogous to subsequent infection of cells in continuous culture. The biological, structural, physical, and serological properties of these two forms of enveloped nucleocapsids, as discussed earlier, are significantly different (45). A summary of the infection processes as it occurs *in vivo* and *in vitro* should be appropriate in an attempt to search for biological correlations to the structural properties and sources of the virus.

During invasion and infection, the polyhedron crystal dissociates in the gut lumen. The enveloped nucleocapsid enters the gut cell by viral envelope fusion with the microvillar membrane (38). The nucleocapsid has been observed in cytoplasm where it may possibly uncoat by unknown mechanisms (53) or interact with the nuclear pore and release the genome into the nucleus (40, 41). The latent period before the appearance of infectious virus has been approximated to be six to eight hours for Ac*M*NPV (45, 46). When assembly of the nucleocapsid occurs in the nucleus, it may have two fates: one possibility is that it may escape the nucleus, enter the cytoplasm, and bud from the surface of the cell into insect hemolymph or into the cell culture medium. This form of the virus has been referred to as a nonoccluded virus (NOV), a plasma membrane budded virus (PMB-NOV), or an extracellular virus. It is also possible that the nucleocapsid may obtain an envelope by *de novo* synthesis (48) in the nucleus and become occluded. Large numbers of polyhedra accumulate in the nucleus finally disrupting the cell.

The basic factors controlling occlusion of enveloped nucleocapsids in the nucleus or budding from the cellular surface are not understood. It has been suggested that the source and/or information in the different forms of the viral envelope may be responsible (41). Release of extracellular virus and occlusion appears to be a time and tissue dependent relationship (45). Nucleocapsids which are assembled before the synthesis of some virus specific protein, perhaps the polyhedrin required for occlusion, are able to escape the nucleus. Those assembled after the synthesis or presence of polyhedrin are occluded.

There are major differences when comparing the biological properties of the two forms of virus. The nonoccluded virus is more highly infectious. For Ac*M*NPV it has an infectious:physical particle ratio of 1:128. The alkali-liberated virus as titrated in TN-368 cells is less infectious, one particle in 200,000 to 240,000 (Table 2).

A preliminary study (65) used comparative bioassays in insects and the polyhedral plaque assay in cell culture for measuring the relative infectivity of extracellular and alkali-liberated virus. The assays were compared both by oral (*per os*) and hemocoelic injection. By oral injection PMB-NOV was 1×10^5 less infectious when compared to infectivity by polyhedral plaque assay. Injection into the hemocoel showed that PMB-NOV is highly infectious and comparable to the levels of infectivity obtained by *in vitro* assay. Since the nonoccluded form of the virus was significantly more infectious by hemocoelic injection than by infection via the midgut (natural route), this result is consistent with the role of NOV or extracellular virus in the nature infection cycle, that is, the secondary infection of tissue.

A comparison of LOVAL (alkali-liberated-virus) infection by *per os* and hemocoelic injection does not support previous assumptions that the alkali-liberated form of the virus is more infectious by injection than feeding. LOVAL was only 9-fold more infectious by *per os* infection as compared to polyhedral plaque assay. Infectivity by oral injection was only 3-fold more efficient when compared to infection achieved by injection into the hemocoel.

SUMMARY

The focus of this conference is to evaluate the molecular virology of baculoviruses relative to some of the more theoretical concerns of their safe use in environmental applications. Some of the ultimate questions for which we are attempting to provide realistic assessments are: can baculoviruses and/or their genomes enter nontarget cells, persist or integrate, or undergo recombination with host DNA or other viral DNA genomes to expand their host range so as to pose any undesirable or unpredictable consequences when used as biological pesticides. As can be seen from the preceding, basic information on baculovirus structure and activity is presently too limited to evaluate, predict, or estimate any potential interaction. Therefore, it is clear that the safety testing required for registration of a baculovirus formulation also does not provide the pertinent information for such assessments.

In a reasonably short period of time, available technology could be employed to investigate the potential interactions identified above in both susceptible and unsusceptible host cells. It is recommended that high priority be given to the study of the molecular aspects of baculovirus structure, biology, and specificity of the infection process. The genetics of baculovirus genomes should be investigated relative to their gene products. Genotypic and phenotypic markers should be identified as those important for the development and application of diagnostic detection probes for surveying the fate of baculovirus activity in any physical or biological environment. Since baculoviruses are genetically and phenotypically complex, this approach will be necessary in order to provide for a more realistic safe use and risk assessment of baculoviruses in their proposed role as biological pesticides.

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DISCUSSION

SMITH: Have these various viruses been tested for the serological relatedness of these granulins?

SUMMERS: Yes. I have discussed only the structural relatedness using peptide mapping techniques. Later, DiCapua and Hoops will review the serological properties.

SMITH: Are granulins and polyhedrins virus-specified?

SUMMERS: I think you can deduce from the peptide mapping studies that they are.

SMITH: When a host makes an antibody to the proteins of the virus, is this a major component of the antibody specificity?

SUMMERS: Against the virus?

SMITH: Yes. Let's say you inject the virus as an antigen into a mouse or rat.

SUMMERS: It will be more appropriate to discuss this subject later.

RAPP: I have a question about the crystalline polypeptide that you say is between 25 and 30 thousand. Is that polypeptide also found in the virion capsid structure? In other words, is it viral-specified?

SUMMERS: There is a protein in the enveloped nucleocapsid with similar mobility to that we described as polyhedrin. We have not determined whether polyhedrin protein is a structural component of the enveloped nucleocapsid.

ULVEDAL: This morning we heard that an insect may harbor several viruses. Do you think with present techniques we could separate these viruses before we use them on a field trial?

SUMMERS: Yes. I am confident that there are a few laboratories that have the ability to evaluate them with respect to their purity before they are used.

ULVEDAL: How pure? One hundred percent pure? What range of purity are we talking about?

SUMMERS: With the techniques we employ, as I have presented to you here, we can identify baculoviruses.

STOLLAR: Is the virus you used to study the DNA and proteins obtained from whole insects?

SUMMERS: Yes, all the structural protein studies that we have done have been with occluded viruses purified from infected insects.

HOLOWCZAK: You seem to indicate that if these viruses are going to be useful as pesticides, they have to be presented as occluded particles. Is there a difference between occluded and nonoccluded viruses in terms of survival?

SUMMERS: The nonoccluded virus does not survive well.

HOLOWCZAK: Purification would be a difficulty if more than one kind of virus could be occluded -- probably impossible.

SUMMERS: Especially if there were mixed populations or strains.

HOLOWCZAK: Have you infected subjects with two related viruses?

SUMMERS: I have decided not to do this until our detection technology is capable of differentiating between the two with techniques to screen for mixed populations.

HOLOWCZAK: Are all these pathways of morphogenesis due to one kind of virus?

SUMMERS: The biological behavior of a baculovirus *in vivo* and *in vitro* is complex. Observations on the structure and biology of baculovirus infection suggest we are working with mixed populations of viruses. At the moment, we do not have sufficient data to answer your question.

RAPP: When you perform a bioassay, you release occluded virus and NOVs *in vivo*. Do you do that by injection or *per os*?

SUMMERS: By *per os* injection and hemocoelic injection. We compared both *per os*. We did not put the virus on any diet; we injected it directly into the gut.

RAPP: Since identification of viruses is going to be critical, especially after field trials, you gave us your favorite scheme for identifying viruses, by their polypeptides. Does DNA hybridization technology correlate with your method of identification?

SUMMERS: I did not give you my favorite scheme for identification. I gave a "most difficult" scheme for identification. I discussed an idealistic approach to evaluate whether serological techniques can be specifically and effectively used with a few highly purified and characterized proteins. We want to test how good our serology can be. Certain DNA studies can be very definitive and can get very dependable results, but very few people are doing restriction-enzyme fragmentation physical mapping. I do not believe we have enough data accumulated to begin to make informed statements.

RAPP: I know there is cross hybridization between baculovirus DNA, and you demonstrated similarities among polyhedrins. What extent of cross hybridization is mirrored by similarities in your tests?

SUMMERS: We are not presently making those kinds of comparisons. We are concentrating on proteins. Perhaps Dr. Harrap can reply to your question.

HARRAP: The extent of cross hybridization we see among *Spodoptera* baculovirus system is mirrored by the sort of serological relatedness among the various isolates we have used.

Identification of Nonoccluded Viruses of Invertebrates

John F. Longworth, Ph.D., and Paul D. Scotti, Ph.D.
DSIR, Entomology Division
Auckland, New Zealand

The term "nonoccluded" is still used to describe those viruses other than NPV, GV, CPV, and entomopoxviruses. The latter viruses become occluded within a proteinaceous crystal at the end of the infection process. The term "nonoccluded" therefore is of little definitive value since it includes such distinct groups as:

- iridoviruses,
- parvoviruses,
- small isometric RNA viruses.

Its use should be discouraged, in favor of using appropriate group names.

The comparative virology of iridoviruses, parvoviruses, and small RNA viruses has been discussed in recent reviews (1-4) and the small RNA viruses affecting bees have been separately reviewed (5).

THE IRIDOVIRUSES

The iridoviruses, together with African swine fever virus, frog virus 3, lymphocystis virus, etc., comprise the icosahedral cytoplasmic DNA virus group. The iridoviruses are commonly associated with invertebrates in an aquatic or a soil environment. The group includes isolates affecting blue-green algae, fungi, and fresh water crustacea. They are large particles,

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up to 180 nm in diameter, containing linear, double-stranded DNA of greater than 100 x 10^6 mol. wt. Over 20 isolates are known from insects and while many have a wide host range within the class Insecta, none are known to infect vertebrates or vertebrate cell lines except that it has been reported that Chilo iridescent virus (CIV) multiplies in cultured viper spleen cells (6). Within field populations of an insect species, infections tend to develop slowly and insect to insect transfer most commonly results from cannibalism; large doses of virus are necessary for successful *per os* infection. Epizootics, leading to marked reduction in insect populations, are rarely observed with iridoviruses and they are not regarded as suitable candidates for applied control.

Several laboratories in the United Kingdom, United States, New Zealand, and Europe have been or are involved with biochemical and serological characterization of these viruses. Their polypeptide, lipid, and nucleic acid compositions are fairly well understood, and studies of the biochemical events in virus replication have also been conducted for some iridoviruses (7).

THE PARVOVIRUSES

Only two invertebrate parvoviruses have been adequately described. These are closely related serologically, their polypeptide composition is identical (2) but there are marked differences in host range. Unlike vertebrate parvoviruses which have three major polypeptides, the invertebrate isolates have four. The linear, single-stranded DNA occurs as + and - forms in separate particles (8). Two principal investigators are working with these viruses, E. Kurstak at Montreal, and D.C. Kelly at Oxford.

Purified densonucleosis virus, originally isolated from the greater wax moth, *Galleria mellonella*, replicated in mouse L cells. Some mouse L cells produced DNV virions, although in lower quantities than can be obtained from insects. The L cell-derived virions caused an infection in *G. mellonella* larvae typical of DNV (9). The majority of the cells, however, were apparently transformed by the *Galleria* virus, and viral antigens were detected by immunofluorescence. Infective virus could not be recovered from these cells.

SMALL ISOMETRIC RNA VIRUSES

Of most interest to this meeting, however, is the rapidly expanding group of known small RNA viruses affecting invertebrates. At the First International Colloquium for Invertebrate Pathology held in September 1976, we listed the properties of nearly 30 such viruses (4) and commented that apart from one or two very obvious similarities between some viruses it was premature to attempt a definitive subgrouping. In the light of new comparative information, particularly that obtained by C. Reinganum when working at T.W. Tinsley's laboratory, at least four logical groupings are now possible. Other individual viruses are clearly distinct, one from another, and are probably the first representatives to be described of other groups. The groupings in Table 1 will be described more fully in a forthcoming paper.

The first group includes Nodamura virus, isolated from mosquitoes in Japan, and *Heteronychus arator* virus from the New Zealand black beetle. These viruses have closely similar biochemical and biophysical properties (10). In particular, both have a divided, single-stranded RNA genome of 1.0 and 0.5×10^6 mol. wt.; with Nodamura virus, both RNA species are necessary for infection. At present, it is not clear whether the RNA species are separately encapsidated; if they are, a quantitative assay would exhibit a two-hit dose-response curve. Both isolates have a wide host range within insects, but Nodamura virus will also infect cultured vertebrate cells and mice. Indeed, this is the only authenticated instance of replication of an insect pathogenic virus in vertebrates. Because of the divided genome, there are very real possibilities of genetic reassortment, not only in contrived laboratory situations, but also in the field, if two such viruses were to infect the same host. Nodamura virus replicates without CPE in *Aedes aegypti* cells and *A. albopictus* cells and can be titrated in mice. The *H. arator* virus will replicate in *Drosophila* cells and causes a cytopathic effect (CPE) after 9 or 10 days, and a titration system is being developed. The *H. arator* virus does not replicate in *A. albopictus* cells, and does not appear to be infective for BHK cells or mouse L cells.

The cricket paralysis virus (CrPV) group includes two serologically related isolates, *Drosophila* C virus and cricket paralysis virus. The *Drosophila* C virus is widely distributed in France, Europe, and the Mediterranean region, and it most commonly occurs as an inapparent infection. Cricket paralysis virus was first isolated from *Teleogryllus commodus* in Australia and is serologically related to *Drosophila* C virus. There are, however, distinct host range differences between the two isolates (N. Plus, personal

TABLE 1. SMALL RNA VIRUSES OF INVERTEBRATES

Virus Group	dia	P	S	Proteins	pH stab	RNA %	RNA mol wts	GS %	Number of isolates in group
Nodamura	29	1.34	135	35,000 plus 2 minor	pH 3.0	-	1.0 0.5	54.6 51.0	2
Nudaurelia β	35	1.298	210	61,000	-	11.0	1.8	53.0	8 plus
Cricket paralysis	27	1.34	167	30,000 32,000 - ?	pH 3.0	28.0	>2.0	38.7	3
Gonometa	32	1.35	180	12,000 29,000 32,000 36,500	pH 5	37.0	-	-	3
Kelp-fly	29	pH 1.43-7	158	29,400 73,000	pH 3.0	-	3.5	37.4	1
Chronic paralysis	30 20 x 40 50 60	1.33	82 100 110 126	23,500	-	-	-	48.0	1

At least 20 other small RNA viruses are known; these are not characterized sufficiently well to allow for comparative treatment. They have been isolated from bees, flies, mosquitoes, termites, and from the crustacea. Thus, nearly 40 such viruses have been described, only two so far from the United States. The vertebrate host range has been tested in detail for only a few of these isolates.

communication). Each virus appears to replicate best in its specific host and only CrPV multiplies in the cricket *Gryllus bimaculatus* (N. Plus, personal communication). CrPV is normally present in *T. commodus* as an inapparent infection and has also been isolated from other cricket species, as well as from Heteroptera and Lepidoptera in several countries. A tissue culture assay method for CrPV is now available (11) and, using a 50% tissue culture infective dose ($TCID_{50}$) assay method, we have begun a survey of the incidence and development of CrPV in cricket populations in New Zealand. Additionally, we will be examining the incidence of this virus in other invertebrates in the same area.

Cricket paralysis virus will replicate in *Drosophila* cell lines 1 and 2, and in *Aedes aegypti* and *A. albopictus* cells. It also replicates in cultured cells of *Porthetria dispar* (N. Plus, personal communication). We have examined the replication of cricket paralysis virus in *Drosophila* cells; the viral RNA apparently directs the synthesis of large precursor polypeptides which are then cleaved into smaller stable proteins. The largest species detected in pulse experiments was around 120,000 daltons, and this peak disappeared during the subsequent chase. The overall pattern of polypeptide synthesis directed by CrPV RNA closely resembles that of poliovirus. It seems logical, therefore, to consider members of the cricket paralysis virus group as true picornaviruses.

The *Gonometa* virus group includes three serologically related viruses (Plus, Reinganum, personal communication): one from bees, one from flies, one from moths--from three different orders of insects in fact. Size, density, sedimentation coefficient and, for the *Gonometa* isolate, polypeptide composition, are all similar to the vertebrate enterovirus group. The *Gonometa* isolate (from East Africa) is one of the few invertebrate small RNA viruses to have been used in insect control -- with success and without obvious effects on vertebrates. Longworth et al. (12) demonstrated that IgM antibodies to *Gonometa* virus were present in a range of domestic and wild animals in the United Kingdom and postulated repeated exposure to low dosages of a virus at that time undescribed in the United Kingdom. The more recent discovery of viruses related to the *Gonometa* isolate in bees and *Drosophila* flies in Europe lends further support to this hypothesis. No IgG antibodies were detected in the test animals which reacted with *Gonometa* virus and the route of exposure to the virus remains open to conjecture.

The *Nudaurelia* β virus group includes serologically related isolates from a range of countries affecting Saturniid and Limacodid moths. These viruses are 35 nm in diameter, 1.30 density, 210 S, and have one major polypeptide. The RNA content (11.0%) is low by comparison with most other small RNA viruses, and the capsid exhibits T_4 symmetry (13); the 240 protein subunits are arranged in trimers with four trimers per icosahedral face. Nudaurelia β virus will replicate in Nudaurelia and Bombyx mori cell cultures (14).

Ecology of Small Isometric RNA Viruses

The presence of antibodies in vertebrates to invertebrate pathogenic viruses needs careful study. The work with Gonometa virus first drew attention to the problem, but has not proceeded further than defining the antibody class involved. Lack of virus hindered this work, but we know that similar studies are in progress at Oxford with other small RNA viruses of invertebrates. The phenomenon is not an isolated one, for in the same study, antibodies were also demonstrated in United Kingdom vertebrates to an RNA virus from the Nudaurelia β group.

In New Zealand, we have examined cattle sera from the site where the RNA virus of H. arator was isolated and failed to find antibodies to that virus using the immunodiffusion technique. However, low levels of precipitating and neutralizing antibodies to CrPV were detected, and this virus had not been recorded in New Zealand at that time. Subsequently, with tissue culture assay methods, we have detected cricket paralysis virus in field crickets from the same site. There may be a purely casual association between the virus present in crickets and antibodies in serum from cattle in the same area, as the cattle must inhale or ingest insect debris frequently, but we plan to explore this further, and to determine the nature of the neutralizing antibodies.

Nodamura virus has only been isolated in the field from mosquitoes, but high levels of neutralizing antibodies were found in pigs and, to a much lower extent, in herons. The infections of the mosquitoes were inapparent, but the virus can cause fatal paralysis in bees, ticks, moth larvae, and mice and is transmissible to mice by infected Aedes aegypti. It replicates in cultured vertebrate and invertebrate cells without CPE (15).

Thus, there is some evidence that there may be an association between some insect small RNA viruses and vertebrates, and this warrants closer study. There is direct evidence that at least one virus -- the Nodamura isolate -- is pathogenic to vertebrates. It is possible that genetic reassortment could occur between divided genome viruses of this type, if they infect the same host. With this in mind it would seem unnecessary to point out the potential hazards of deliberately using such viruses in a spray program to control insects, yet this has happened -- against *Gonometa* in East Africa and against *Darna trima* in Borneo and Sabah. No ill effects have been reported, but then no survey of other invertebrates and vertebrates in the environment was made.

Small Isometric Viruses as Possible Contaminants in Baculovirus Preparations

It is appropriate to consider the possible contamination of baculovirus preparations with a small isometric virus. It has been shown recently (16) that at least one such virus will coinfect *Autographa californica* together with its specific baculovirus. In fact, one 40 nm virus replicated in nuclei in *A. californica* larvae or in *Trichoplusia ni* cells equally well whether or not the baculovirus was replicating in the same nucleus. This baculovirus of course is currently a candidate for development for insect control. In *Heliothis zea*, a parvovirus and a picornavirus have only recently been isolated from laboratory cultures in the United Kingdom (Tinsley, personal communication); the parvovirus is serologically related to *Galleria* parvovirus, and the picornavirus is related to cricket paralysis virus.

Small isometric RNA viruses are more common in insects than has been supposed, and may pose real problems in baculovirus preparations. However, where the properties of the likely contaminant are known, it is possible to devise rapid and sensitive assays to monitor for its presence, and, if necessary, it is not difficult to eliminate such contamination from baculovirus preparations by relatively simple purification procedures.

It is, of course, desirable to propagate the candidate virus in a disease-free laboratory colony of the specific host, but it is pertinent to stress here that while most of the invertebrate picorna-like viruses can kill their hosts, many of them occur in nature as inapparent infections. Nodamura virus does not kill its *Culex* mosquito host (17); Kelp-fly virus (18) was only isolated by passaging through moth larvae, extracts of apparently healthy Kelp-flies which did not contain detectable viruses. Many bee viruses only kill their host when injected in high concentration (5). We have detected an average of less than 100 infectious particles of CrPV per cricket from field samples--a level which would defy detection by standard serological techniques. One cannot assume that absence of mortality in a laboratory insect colony necessarily means absence of a small RNA virus.

It is practicable to reduce the level of a contaminating small isometric RNA virus drastically during purification of baculovirus polyhedra; the S_{20} of polyhedra is very high, and they rapidly reach their isopycnic point at around 54% w/w sucrose. Though the RNA virus has a higher density, its S_{20} is so low that it is well separated on the gradient. We have performed experiments to show that contamination by CrPV can be drastically reduced in a baculovirus preparation by simple extraction procedures and reduced below the level of detection of our tissue culture assay by one cycle of zonal centrifugation (19).

Future Research Needs with Small Isometric Viruses of Invertebrates

1. The small isometric viruses which have been described should be fully characterized as a basis for reliable comparative studies. This applies particularly to the small RNA viruses.
2. The *in vitro* and *in vivo* host range of small isometric viruses should be established to allow an assessment of the likelihood of hazard not only to vertebrates, but to beneficial invertebrates.
3. The significance of antibody responses in domestic and wild animals to small RNA viruses of invertebrates needs to be determined. Do all the instances involve IgM only, and does this imply limited casual exposure to antigen, rather than infection?
4. Can mosquitoes acquire infective doses of invertebrate small isometric viruses and transmit these to vertebrates?
5. Can the divided genome RNA viruses exchange genetic information? If so, how common are these viruses; do they present a hazard?
6. We need quantitative assays, particularly for small isometric viruses which do not cause CPE.
7. Does the possible presence of small isometric viruses in commercial baculovirus preparations constitute a hazard which merits including additional safeguards in the production process? There appear to be three alternatives:
    a. Assuming that present bioassay checks in the development and production process, including batch tests, give sufficient protection already.
    b. Adding specific tests such as radioimmunoassay or tissue culture assays for viruses which have been isolated previously from the candidate insect.

c. Instituting simple purification procedures in the production process which would exclude possible contaminant viruses.

Finally, it should be pointed out that there are less than ten people who are engaged in research on small isometric viruses of invertebrates on a full-time basis; these workers are all in the United Kingdom, Canada, France, Australia, and New Zealand.

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DISCUSSION

LONGWORTH: Do you have additional information on the 40 nm isometric virus-like particle observed in a mixed infection with Autographa NPV?

SUMMERS: We know it has one structural polypeptide, but the density of 1.24 gm/cc is a mystery to us. We have not yet confirmed whether it is a DNA or RNA virus.

STOLLAR: These small RNA viruses look like agents with which care will have to be exercised if they are to be used as contaminants in any viral pesticides. Does Nodamura kill suckling mice?

LONGWORTH: Perhaps Dr. Shope can answer this question.

SHOPE: Nodamura virus kills baby mice by intracerebral and intraperitoneal inoculation, but it would not be transmissible to adult mice, or at least it does not make them very sick. I am not certain whether antibody studies have been conducted using adult mice.

STOLLAR: What did you say about replication in vertebrate cells in culture?

LONGWORTH: There was no CPE in invertebrate or vertebrate cells *in vitro*.

STOLLAR: There are different size RNAs. Is there any suggestion of different size particles?

LONGWORTH: No, the size is homogenous in all respects.

SMITH: I am encouraged by your approach of looking at those animals in the field that are probably most directly exposed to these agents. Have insectivorous species such as bats been monitored at all? Have you found evidence of replication of these viruses?

LONGWORTH: As far as I know there is no evidence of replication at all. Using a radioimmunoassay, we looked for antibodies to New Zealand NPV in birds that prey on caterpillars. The bird feces contained 18 percent by weight of inclusion bodies, having gone through unaffected. Those 26 birds have no antibodies even though it was a major epizootic.

SUMMERS: What if you used a nonoccluded virus?

LONGWORTH: This work was not done. The sera are available, as I understand it. Kalmakoff, who is the principal investigator, is continuing the work.

HARRAP: Quite a bit of work is being done at Oxford on the antigen-antibody reactions with two of these viruses, Nudaurelia β and Darna trima, but it has not really appeared in a published form. I will summarize using Darna trima virus (34 nm in size) and the Nudaurelia β virus. We had reactions to one or the other, and sometimes to both -- sheep, cats, pigs, humans, and with some African animals, for example, lions, wart hogs, water buffalo, and two zebras. All Antarctic sera that we tested from the British Antarctic survey have been negative. We are now looking at birds, whose sera we have not yet examined. The aim of the project is not to screen vast numbers of sera, but to identify for certain what the antibody type is or, more importantly, try to identify the agent in the animal that elicits the antibody response that will react with our insect virus. We have detected particles in sera by electron microscopy, especially with sera from a particular flock of sheep in Oxfordshire. The particles are the right size for picorna viruses. We are hopeful that we can make isolations of virus from the sera. The antibody level seems to increase and the particles disappear in individual sheep as we bleed them over a period of time. So, it is a matter of choosing the right stage for attempts at virus isolation.

PAGANO: After the Nodamura virus replicates in suckling mice, has it been repassed in mice? Does it gain virulence?

LONGWORTH: I cannot recall whether this has been done. I can refer you to the publications of Newman or Brown.

SHOPE: I worked with Nodamura virus in 1959 and we passaged it in baby mice, and it maintained virulence on passage, 10 or 12 passages. As far as I know, it did not gain virulence, as injected by the intraperitoneal route. It did not kill adult mice because we immunized our mice with high passage level virus, nor did it kill adult mice by intracerebral inoculation.

Biology of Cytoplasmic Polyhedrosis Viruses and Entomopoxviruses

Robert R. Granados, Ph.D.
Boyce Thompson Institute
Cornell University
Ithaca, New York

Although the major attention during this symposium will be focused on the baculoviruses, it was felt that some time should be devoted to the discussion of cytoplasmic polyhedrosis viruses (CPVs) and entomopoxviruses (EPVs). Indeed, there is not only interest in some countries in developing these viruses as viral pesticides, but at least one CPV has been registered in Japan for use against a forest insect pest.

Unlike baculoviruses, CPVs and EPVs should be more familiar to most virologists since these viruses have many properties in common with plant and/or vertebrate viruses. My discussion will be limited to presenting a brief overview of the current knowledge of these two insect virus groups.

For the purpose of this discussion, I will use the term "occlusion body" (OB) to designate the proteinaceous, virus-occluding structures commonly referred to as polyhedral inclusion bodies, polyhedra, inclusion bodies, spheroids, virus-containing inclusions, granules, capsules, and so on. The term "OB" was coined by Dr. Brian Federici (1) a few years ago in order to avoid confusion with the term "inclusion body" which is used by vertebrate virologists to denote a very different structure.

The international committee on taxonomy of viruses recently recommended that a genus comprising CPVs be established within the family Reoviridae

(2). Viruses in this family possess genomes consisting of several (10-12) segments of double-stranded (ds) RNA with molecular weights ranging from $0.3-3 \times 10^6$, all ds-RNA pieces are encapsidated within a single virus particle, and the virion has an isometric capsid (double protein shell) with icosahedral symmetry, 60-80 nm in diameter (Table 1). CPVs differ from other members of the Reoviridae family in that arthropods (primarily insects) are the only known hosts, and virions may be occluded in proteinaceous OBs. Recently, a CPV was found in a freshwater daphnid (Crustacea) (3).

TABLE 1. CYTOPLASMIC POLYHEDROSIS VIRUS
(FAMILY REOVIRIDAE)

Cryptogram:
[R/2 : 13- 18/25-30 : So/S : I/O]

Main characteristics:

1. Genome has about 10 pieces of double-stranded RNA.
2. Total genome molecular weight of $13-18 \times 10^6$ daltons.
3. Virions are 50-60 nm in diameter with projections at vertices of icosahedron. Two-layered capsids.
4. RNA-dependent RNA polymerase present in virion.
5. Particles may be occluded in crystalline protein occlusion bodies.
6. Initially they infect only the cells of the midgut but may spread to other tissues.
7. Host range: Lepidoptera, Diptera, Hymenoptera, Neuroptera, and Crustacea.

The CPV of the silkworm, *Bombyx mori*, is the most thoroughly studied virus of this group. The particles consist of two concentric icosahedral capsids. The outer capsid has a diameter of 65 nm and the inner shell a diameter of approximately 45 nm. The outer shell of the virion consists of 12 pentagonal capsomeres, localized at the vertices of the icosahedron. The capsomeres are in the shape of hollow pentagonal disks with an outer diameter of 20 nm and inner diameter of 5 nm. Each capsomere has a hollow segmented projection. At the end of these projections, structures measuring 12 nm in diameter have been observed. Tubular structures connect the outer capsid shell to the inner shell at the 12 vertices.

The replication cycle of CPVs has not been critically examined in synchronously infected cells. Following larval ingestion of OBs, these crystals are dissolved by the gut juices in the lumen, thereby releasing numerous virus particles. Kobayashi (4), in a study designed to determine the mechanism of *B. mori* CPV penetration into host intestinal cells maintained *in vitro*, observed that the first stage of entry was the attachment of viral projections to the cell surface. Within 10 minutes postinoculation, the attached virions released viral core material into the cells. This was suggested by the presence of empty capsid shells on the cell surface and by the presence of dense material in the cytoplasm directly beneath empty particles. Kobayashi concluded that the viral core substance was released as a filament and injected in much the same manner as a bacteriophage. Phagocytosis did not appear to play a part in virus penetration. Several investigations have indicated that after internalization of the viral genome, transcription of the parental RNA occurs in the nucleus. Virus transcription in the nucleus is subsequently followed by protein and ds-RNA synthesis, virus assembly, and occlusion in the cytoplasm.

Nonoccluded virions may represent more than 70% of the total number of virus particles in an infected insect (5). Preliminary studies (6) suggest that the biochemical properties of occluded and nonoccluded *B. mori* CPV particles are similar. *B. mori* particles extracted from OBs (occluded virions) have a density of 1.43-1.48 g/cm^2 in CsCl and contain five structural polypeptides, as determined by polyacrylamide gel electrophoresis. Nonoccluded virus particles have the same RNA components, structural proteins, and density as occluded virions. It has not been determined if the biological properties of these two "forms" of CPV are similar. Insofar as the OB is concerned, *B. mori* CPV has a major polypeptide with a molecular weight of 27,100 daltons and contains a covalently attached carbohydrate moiety.

Electrophoretic analysis of the proteins of virus particles and OBs has been shown to be a useful tool in differentiating between CPVs. However, a more powerful tool for the identification of CPVs is an analysis of the number and/or size of the RNA genome segments following fractionation on 3% polyacrylamide gels (7). For example, Payne (7) reported that when the ds-RNAs of 33 CPV isolates were examined by gel electrophoresis, some viruses had identical profiles, and he was able to group 29 of the isolates into 11 virus "types" which were identified by major differences in the mobilities of the RNA segments. Four of the CPV isolates contained more than 10 RNA segments, and these were interpreted as mixtures of two viruses. Gel analysis of viral RNAs can be made without difficulty and from impure samples if necessary (7). It would appear that adequate tools for the identification of CPVs are currently available.

Most of the research on CPVs has been conducted with virus produced in living insects. Only in the last 2 years has there been some effort in developing tissue culture techniques (8, 9). In our laboratory, we have been able to infect four insect cell lines with CPV -- the fall armyworm, *Spodoptera frugiperda*; the cabbage looper, *Trichoplusia ni*; the salt marsh caterpillar, *Estigmene acrea*; and the gypsy moth, *Porthetria dispar*. An immunofluorescence assay for CPV in cell cultures was developed, and the ultrastructure of the replication cycle *in vitro* was studied (8). However, much more work is still needed in this area before the routine use of insect cell cultures in CPV research can be realized.

In vivo specificity studies have shown that CPVs have a wider host range than EPVs or baculoviruses. Approximately 175 insect species (mainly Lepidoptera) are known to have a CPV infection (10), and recently a CPV-type virus was reported in the freshwater daphnid, *Dimocephalus expinosus* (3). This crustacean was also infected with an iridovirus-type virus. At present, at least three types of viruses commonly found in insects (two baculoviruses, one CPV, and one iridovirus) have now been reported in the Crustacea. These recent findings reemphasize the need for more research directed toward determining the kinds of viruses which occur in Crustacea and other invertebrates.

There is little information on the effect of CPVs on other invertebrates or vertebrates. No apparent infection resulted following inoculation of turkey embryos (11) or four mammalian cell lines (14-HeLa, human amnion cells, porcine kidney, and mouse sarcoma) with *B. mori* CPV. In our laboratory, we were unable to infect (based on CPE using light and electron microscopy) four mosquito cell lines and two vertebrate cell lines (HeLa and L-929 mouse fibroblast) with a CPV from *T. ni* (8).

The potential of CPVs as viral pesticides is good. There are several CPVs that have given reasonably good control of their hosts (Table 2). At least one has been registered for use against the pine caterpillar (*Dendrolimus spectabilis*) and is commercially produced under the trade name Matsukemin (12). The safety of this CPV was tested using mice, rabbits, chick embryos, hamsters, and fish. No pathogenicity was observed in any test (13). A major obstacle in the development of CPVs as viral insecticides is their taxonomic relatedness to Reovirus and other vertebrate viruses. If safety testing continues to yield negative results (i.e., no adverse effects), there may very well be a renewed interest in the study of these viruses.

TABLE 2. SOME CYTOPLASMIC POLYHEDROSIS VIRUSES (CPVs) WHICH HAVE BEEN TESTED AS BIOLOGICAL CONTROL AGENTS

Virus	Target insect	Country
Lymantria CPV	*Lymantria fumida*	Japan
Trichoplusia CPV	*Trichoplusia ni*	United States
Thaumetopoea CPV	*Thaumetopoea pityocampa*	France
Dendrolimus CPV*	*Dendrolimus spectabilis*	Japan
Lymantria CPV	*Lymantria dispar*	Japan

*Registered in April 1974; commercial name: Matsukemin

I would now like to turn my attention to a discussion of entomopoxviruses. These viruses are members of the genus *Entomopoxvirus* (EPV) in the family Poxviridae (2). Viruses in this family are large, brick-shaped, or ovoid virions 250 x 350 nm in dimension (Table 3). The virions have a lipoprotein envelope with tubular or globular protein structures on the outer surface, enclosing one or two lateral bodies and a core, containing the genome. The genome consists of a single molecule of double-stranded DNA of molecular weight 130-240 x 10^6 daltons. Virions contain more than 30 structural proteins and several viral enzymes. Insects are the only known hosts of insect poxviruses although some vertebrate poxviruses are known to be vectored by insects in a nonpersistent manner. Four orders of insects, Lepidoptera, Coleoptera, Diptera, and Orthoptera, are affected, with most viruses isolated from Lepidoptera and Coleoptera.

Unlike other members of the Poxviridae, EPVs can be subdivided into three subgenera based on the morphology of the virion (14) (see Figure 1). Subgroup 1 (= subgenus C) has brick-shaped virions with two lateral bodies and a biconcave core. These viruses affect species in the order Diptera (e.g., mosquitoes and chironomids), and morphologically the virions are very similar to vertebrate viruses. Subgroup 2 (= subgenus A) has ovoid virions with one lateral body and a unilateral concave core. These viruses affect the coleopteran (beetles) species. Subgroup 3 (= subgenus B) has ovoid virions with a sleeve-shaped lateral body and cylindrical core. Lepidopterans (moths) and orthopterans (grasshoppers) are hosts for this virus subgroup.

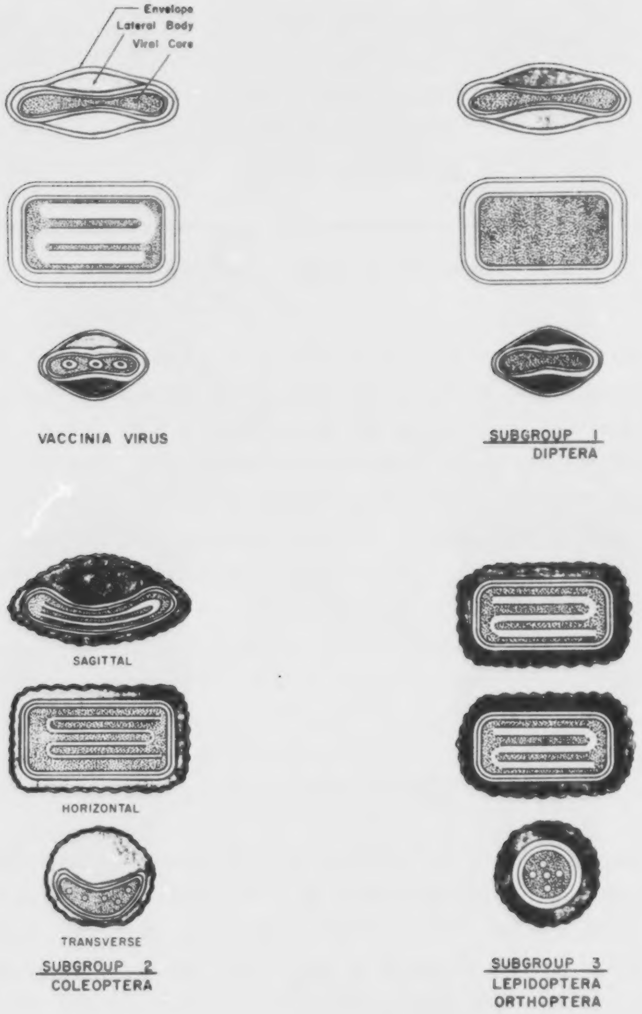
Envelope
Lateral Body
Viral Core
VACCINIA VIRUS
SUBGROUP 1
DIPTERA
SAGITTAL
HORIZONTAL
TRANSVERSE
SUBGROUP 2
COLEOPTERA
SUBGROUP 3
LEPIDOPTERA
ORTHOPTERA

Figure 1. Diagrammatic representation of 3 entomopoxviruses and vaccinia virus in 3 planes of symmetry.

TABLE 3. INSECT POXVIRUS SUBGROUP (FAMILY POXVIRIDAE)

Approved name:

Entomopoxvirus

Cryptogram:

[D/2 : 140-240/5-6 : Xo/* : I/O]

Main characteristics:

1. Genome consists of a single molecule of double-stranded DNA. Molecular weight 140-240 x 10^6 daltons.
2. Brick-shaped or ovoid virion, 170-250 x 300-450 nm.
3. Virions contain at least four enzymes found in vertebrate poxviruses.
4. Particles may be occluded in crystalline protein occlusion bodies.
5. Three subgenera based on morphology of virions.
6. Host range: Lepidoptera, Coleoptera, Diptera, and Orthoptera.

During the course of viral replication, many EPVs of all three subgroups produce OBs as well as antigenically distinct spindle-shaped bodies. These "spindles" are always virus-free and are proteinaceous in nature. A somewhat analogous situation occurs with some, but not all, vertebrate poxviruses. These poxviruses (e.g., strains of cowpox) produce A-type inclusions that are distinctly different from the so-called B-type inclusions (viroplasms), the site of viral multiplication. Depending on the virus type and strain, these A-type inclusions have distinctly different properties and may either contain virions or be completely free of them.

The replication cycle of at least two EPVs (*Melolontha melolontha* and *Amsacta moorei*) has been carefully studied in living insects, and *A. moorei* EPV is currently being studied in detail in BTI-EAA cell cultures. The entry of an EPV (*A. moorei*) into a host insect has been studied *in vivo* and *in vitro* (15, 16). After ingestion of OBs, the gut juices dissolve the protein crystal, thereby releasing virion into the lumen of the midintestine (15). Within 2 hours postinoculation, fusion of the viral envelope and microvillus membrane occurs and virus cores plus lateral bodies enter into the microvilli. The viral cores then descend down the microvilli, enter the cell cytoplasm, and initiate an infection. Under cell culture conditions, virus uptake occurs by viropexis (phagocytosis). Virus synthesis and assembly occur in the cytoplasm in viroplasmic areas (or B-type inclusions). EPV undergo a maturation process from immature forms to mature forms, and these virions may be released from infected cells or occluded within OBs. Except for minor details, the replicative cycle of EPVs and vertebrate poxviruses is similar. This was further confirmed by recent studies which showed that rifampicin (an inhibitor of vertebrate poxvirus multiplication) was also effective against A. moorei EPV in cell cultures (17). In cultures treated with 100 μg/ml of rifampicin, virion formation was blocked, and viral membranes with irregular contours accumulated around "viroplasmic areas." This inhibition was reversible.

EPVs and vertebrate poxviruses have numerous properties in common (Table 4). Some notable differences include the following:

1. Host range.
2. Length of the replication cycle is much longer for EPVs.
3. Insect viruses do not appear to reactivate as do vertebrate viruses (D. Roberts, personal communication).
4. The DNA G+C ratios of EPVs appear to be 30 to 40% lower than G+C values for vaccinia DNA (18).

Recent studies (19) have been aimed at characterizing and identifying several EPVs from all three subgroups. The molecular weight of DNA from five EPVs was determined by electron microscopy and compared with vertebrate virus DNA (vaccinia). Lepidopteran EPV-DNA (135×10^6) was approximately equal to vaccinia DNA (132×10^6) in molecular weight. The molecular weight of dipteran and coleopteran EPV-DNA (200×10^6) was approximately 50% greater than vaccinia DNA. The orthopteran EPV-DNA (123×10^6) molecular weight was less but similar to the lepidopteran viruses. The dipteran and coleopteran EPV genomes were similar to those reported for fowlpox DNA. Therefore, the two DNA size classes found in EPVs are also found in vertebrate poxviruses.

Langridge (personal communication) has also examined the structural polypeptides of virus particles of two lepidopteran EPVs, one orthopteran EPV, and vaccinia, by SDS acrylamide gel electrophoresis. Each EPV contained approximately 30 peptides and differences in peptide distribution in the gels (based primarily on molecular weight) indicated that EPVs are considerably different from each other and vaccinia.

TABLE 4. PROPERTIES OF INVERTEBRATE AND VERTEBRATE POXVIRUSES

Property	Host	
	Invertebrate	Vertebrate
Size (nm)	350 x 250	325 x 200
Host Range	Insects	Vertebrates
Replication Cycle (hrs)	18-24	6-8
Buoyant Density (Virus, gm/cc)	1.262	1.279
Inclusions (= occlusion bodies)	+	-(+)
RNA Polymerase	2	2
DNAse	2	2
Nucleotide Phosphohydrolase	1	1
Nucleic Acid	DNA (ds)	DNA (ds)
DNA Molecular Weight (Daltons x 10^6)	135-200	132-200
Buoyant density (DNA, gm/cc)	1.678	1.692
TM (°C)	77-78	83
G + C (Mole %)	18-22	34
Rifampicin Sensitive	+	+

There are several insect cell lines susceptible to *A. moorei* EPV — *Heliothis zea*, IPLB-1075; several *Porthetria dispar* lines; and *Estigmene acrea*, BTI-EAA). The best system is the BTI-EAA cell line which was derived from *E. acrea* hemocytes and grows in suspension (16). Only *A. moorei* EPV has been grown in cultured cells, and there is an obvious need for more work in this area of EPV research. BTI-EAA cells are highly susceptible to AMEPV and a plaque assay system was recently established which is similar to the agar overlay assay developed by Dr. H.A. Wood for *A. californica* NPV. Two AMEPV virus clones have been isolated using the plaque assay technique, and these will be compared to the "wild" virus type.

Synthesis of AMEPV proteins during infection of BTI-EAA cells, by following incorporation of ^{35}S labeled methionine into viral peptides, is currently being investigated (W.H.R. Langridge, personal communication). Separation and identification of viral peptides by PAGE autoradiography allowed the identification of time postinoculation when viral peptides were synthesized, cleaved, or degraded.

As indicated earlier, insects are the only known hosts of EPV. *In vivo* transmission tests have shown that EPV are only cross transmitted to related hosts and current information indicates that these viruses have an *in vivo* specificity similar to the baculoviruses. As is the case with CPVs, the effect of EPVs on vertebrate animals has received little attention. At least three EPVs (*M. melolontha* EPV, AMEPV, and *Choristoneura fumiferana* EPV) have been tested on rats and mice (20). Virus and/or OBs were administered by feeding or inoculation. No adverse effects were observed in any test with these EPVs. Caged wild mammals (*Microtus pennsylvanicus*, *Peromyscus maniculatus*, *Clethrionomys gapperi*) and laboratory mice exposed to aerial spray of field-applied *C. fumiferana* EPV showed no adverse effect (20).

In vitro studies have shown that EPVs will not readily infect insect cell lines. Only *A. moorei* EPV can be grown *in vitro*. All attempts to infect various insect cell cultures with *E. auxiliaris* and *Goeldichironomus holoprasinus* EPVs have failed. We have inoculated HeLa and mouse L-929 cells with *A. moorei* EPV, and no cytopathic effect (CPE) was observed by phase or electron microscopy. Most of the virus did not become cell-associated; however, occasionally a few virions were observed within the phagocytic vacuoles but never in the cytoplasm *per se*.

Studies by Roberts and Campbell (21) have shown that centrifugation (1000 x G - 1 hr) of *A. moorei* EPV onto various vertebrate and insect cell lines will cause enhancement of cell fusion and/or death of cells. This effect will *not occur* if cultures are inoculated without centrifugation or if centrifugation without virus is carried out. Studies to resolve this phenomenon are in progress.

Several EPVs have been used experimentally in small-scale microbial control programs (Table 5). Reasonable success has been achieved with some of these viruses, and some researchers feel that EPVs should have the same potential for use in biological control as the baculoviruses.

In summary, viruses are generally considered among the safest of all microbial agents proposed for insect control. However, work on CPVs and EPVs has been hampered by fears of their safety since morphological and biochemical similarities to vertebrate viruses exist. Although current data suggest that CPVs and EPVs are safe, much more research is needed in order to develop and use these viruses in a safe and effective manner.

TABLE 5. ENTOMOPOXVIRUSES (EPVs) WHICH HAVE BEEN TESTED AS BIOLOGICAL CONTROL AGENTS

Virus	Target insect	Country
Wiseana EPV	*Wiseana* spp.	New Zealand
Melolontha EPV	*Melolontha melolontha*	France
Choristoneura EPV	*Choristoneura fumiferana*	Canada
Anomala EPV	*Anomala cuprea*	Japan

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DISCUSSION

COLLINS: You did not mention specifically whether both the CPVs and the EPVs have been tested *in vitro* for their ability to affect animal cells.

GRANADOS: I mentioned in the early part of my talk that Dr. Kawanishi is going to include this in his talk tomorrow morning.

COLLINS: With the radioimmunoassay that is now available with CPVs, has anyone yet tried to define group or type antigens for individual CPVs?

GRANADOS: Most of the work in this particular area has been conducted by Dr. Payne at Oxford. Perhaps Dr. Harrap could elaborate on this.

HARRAP: Dr. Payne has done some radioimmunoassay work; however, we were so pleased with RNA profile identification that we did not pursue the serology too much.

STOLLAR: Do you have any knowledge of the occlusion protein of the pox viruses? Is it a simple one, like that of baculoviruses?

GRANADOS: The major polypeptide is in the range of 27 to 30 thousand in its molecular weight, similar in size to that found in baculoviruses. In addition, there is an alkaline protease; however, this work is still not complete.

PART III
VIRUSES: RECENT ADVANCES

Recent Advances in the Antigenic Characterization of Nuclear Polyhedrosis Viruses*

Richard A. DiCapua, Ph.D., James E. Peters,
and Philip W. Norton
University of Connecticut, Storrs

With the current interest in nuclear polyhedrosis viruses as biological insecticides, the need for specific and appropriate serological assays for the identification and quantification of these viruses has become increasingly important. Specific and reproducible serological data concerning these viruses will be required for environmentally sound decisions about their utilization.

The first comprehensive serological characterization of the nuclear polyhedrosis viruses (NPV), following the introduction of serological assessment of insect viruses by Aoki and Chigasaki in 1921, was initiated in the laboratories of Krywienczyk and Bergold (1, 2). Through their work, utilizing complement fixation and immunodiffusion assays, three distinct serological categories were proposed: 1) the capsule viruses of Lepidoptera, 2) the polyhedrosis viruses of Lepidoptera and 3) the polyhedrosis viruses of Hymenoptera (1, 2). More recently, Norton and DiCapua (3) and Tignor et al. (4), utilizing complement fixation, hemagglutination-inhibition, and immunodiffusion assays, have independently demonstrated a serological relationship between the polyhedrosis viruses of Lepidoptera and Hymenoptera (i.e., shared antigenicity between _L. dispar_ and _N. sertifer_ NPV polyhedrins).

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More exhaustively, DiCapua and Norton (3) have identified, as indicated in Table 1, at least one common baculovirus polyhedrin group-specific antigen (BP-gs) in 15 different nuclear polyhedrosis and granulosis viruses (in press).

TABLE 1. NUCLEAR POLYHEDROSIS AND GRANULOSIS
(BACULO)VIRUSES CONTAINING BP-gs ANTIGEN(S)

NPV		GV
A. caja	*N. teadea*	*m. disstria*
A. californica	*O. leucostigma*	*P. brassicae*
B. mori	*P. idaeusalis*	
H. pseudotsugata	*P. includens*	
H. zea	*T. ni*	
L. dispar	*S. frugiperda*	
N. sertifer		

Our laboratory is currently directing its efforts toward the isolation of type-specific antigens (ts) from several nuclear polyhedrosis viruses, with particular interest in *L. dispar* NPV. To date, neither the identification nor isolation of a type-specific antigen(s) (i.e., an antigen eliciting an antibody [monospecific] which will react only with the homologous nuclear polyhedrosis virus) has been reported. It is, therefore, the purpose of this communication to report the presence of several type-specific antigens of *L. dispar* NPV (B-Ld-ts) and the methods used to identify these antigens.

The isolation of NPV proteins (i.e., containing both group- and type-specific antigens) continues to involve polyhedron dissolution by the method of Bergold or modifications thereof. Prior to dissolution, we pretreat the inclusion bodies with 1% SDS, 3M urea and utilize sucrose gradient centrifugation to remove nonviral components [modifications of Hedlund (5), McCarthy and Liu (6)]. The alkaline dissolution is carried out by suspending 5 mg/ml of polyhedral inclusion bodies in 0.008M or 0.05M Na_2CO_3 at 37°C with variations in dissolution duration of 3, 5, 30, or 60 minutes. The dissolution period is terminated by the addition of an equal volume of

0.05M TRIS buffer (pH 8.5). The suspension of virus and polyhedrin is then centrifuged twice to provide a final supernatant devoid of intact virions. Evidence from our experiments and those of Krywienczyk and Bergold (1, 2), Beaton and Filshie (7), and Summers (8) indicates this alkaline dissolute contains solubilized virion components as well as polyhedrin.

The 0.05M Na_2CO_3, 37°C, 5-minute alkaline dissolute of L. dispar NPV was then subjected to isolectric precipitation (IP) for the purpose of obtaining aliquots of different proteins which could then be utilized as immunogens and assay antigens. The IP procedure, a modification of that developed by Dr. Charles Reichelderfer, University of Maryland (personal communication), employs sequential pH adjustments utilizing HCl to reduce the pH stepwise. The precipitate produced after each pH adjustment was collected, washed thoroughly, and recollected by centrifugation. These pH fractions were then used as the immunogen or test antigen reagent identified above.

Immune sera were produced against these immunogens by a modification of the method of Vaitukaitus (9). Inocula were administered, in complete Freund's adjuvant, by multiple-site intradermal route in the shaved backs of New Zealand white rabbits. The rabbits were boosted at 2-week intervals and bled 8-10 weeks post-initiation of the inoculation sequence. The resulting immune sera were then used for various assays, including immunodiffusion, immunoelectrophoresis, crossed immunoelectrophoresis, and mixed hemagglutination. Control rabbit and guinea pig antisera, with specificity for noninfected hemolymph, hemocytes, ovarian culture cells, and Bacillus thuriniensis, do not produce, in these assays, cross reactivity to polyhedrin or virions released from the "clean" polyhedra.

In our earlier antigenic studies of L. dispar and A. californica NPV polyhedrins, we utilized the 60-minute dissolution period to acquire immunogen for antiserum production in rabbits, guinea pigs and chickens (2, 10). In collaboration with Dr. William McCarthy, Penn. State University, we were able to demonstrate, by PAGE analysis, 1) the presence of alkaline protease degraded polyhedrin components which are eliminated by decreasing dissolution times to 3-5 minutes with an immediate pH adjustment to 8.5, and 2) which are absent from L. dispar and A. californica polyhedrins of cell culture derived polyhedra, independent of dissolution time or carbonate concentration (McCarthy and DiCapua, submitted for publication, Intervirology). Thus our studies on the extent of the presence of BP-gs include antisera raised against homologous and heterologous "degraded" and nondegraded host and nondegraded tissue culture derived polyhedrins (Table 1). These anti-

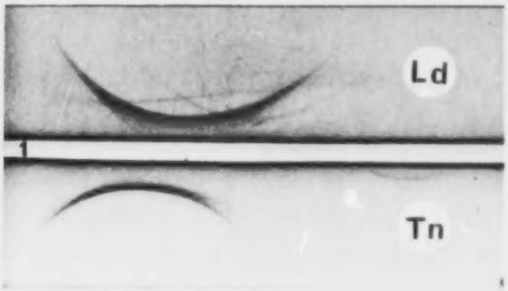

Figure 1. Immunoelectrophoresis. L.d. - *Lymantria dispar* NPV polyhedrin; T.n. - *Trichoplusia ni* NPV polyhedrin; 1 - Rabbit antisera to *L. dispar* NPV polyhedrin. The polyhedrins used as assay antigen and immunogen were collected after 30 minutes dissolution in 0.05 M Na_2CO_3.

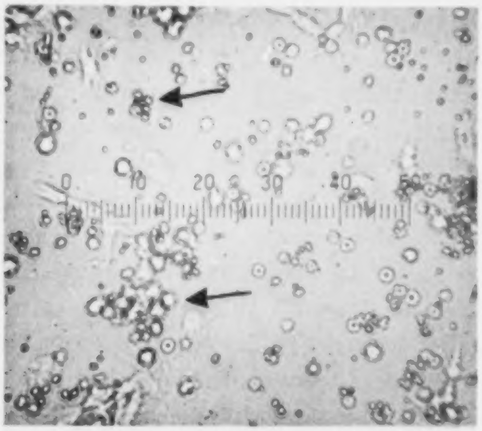

Figure 2. Indirect mixed hemadsorption. Arrows indicate rosette formation absent in assays utilizing noninfected hemocytes (not shown).

sera, produced via an immunization schedule previously published by Norton and DiCapua (2), as well as by the Vaitukaitus modification (9), produce quantitative but not qualitative BP-gs results.

Through the use of immunoelectrophoresis [modification of Grabar and Williams (11)] and crossed immunoelectrophoresis (12), we are able to identify at least five distinct antigens associated with *L. dispar* NPV, four of which, by comparative immunoelectrophoresis, are type specific (i.e., B-Ld-ts). In addition, three of these *L. dispar* NPV type specific have electrophoretic mobilities slower and the fourth, faster than the group antigen (i.e., BP-gs). This has been determined by comparison with alkaline dissolution preparations of *T. ni* (Figure 1), *O. leucostigma*, *N. sertifer*, *S. frugiperda*, and *H. zea* NPVs. We have also identified differences in the electrophoretic mobility of the groups antigen from these viruses. Adsorption of *L. dispar* polyhedrin preparations with antisera produced against *S. frugiperda* and/or *S. exempta* polyhedrins produces, by immunoelectrophoresis, precipitates with mobilities analogous to the type-specific antigens of, but not the group-specific antigen of, *L. dispar*. This is in absolute accordance with the literal definition of type-specific antigenicity.

Utilizing sucrose gradient isolated virion preparations, we can demonstrate that several of the slow mobility type-specific antigens of the polyhedrin of *L. dispar* NPV are associated with the virion and, probably, the nucleocapsid. However, since we can also demonstrate group antigenicity in the virion preparations, this may be due to polyhedrin contamination as observed by Summers in his experiments. Consequently, the question of true shared antigenicity between virions and polyhedrin vs. contamination remains unresolved.

The last technique we wish to discuss is that of mixed hemadsorption (MHA) chosen because of its sensitivity for the detection of nanogram levels of antibody and its application to cell culture systems (13, 14). The *indirect MHA* combines infected hemocytes, taken from 2nd instar *L. dispar* 48 hours post-*per os* infection, with the gamma-globulin fraction of the rabbit anti *L. dispar* NPV polyhedrin. This mixture is incubated at 37°C for 3 hours (to produce a potential agglutinate) and then mixed with guinea pig erythrocytes covalently linked to goat anti-rabbit globulin to produce the rosette formation (Figure 2) which is indicative of the presence of infected hemocytes. The *direct MHA* directly combines infected hemocytes with guinea pig erythrocytes covalently linked to the rabbit anti *L. dispar* NPV polyhedrin. The major control for either assay (i.e., absence of rosette formation) substitutes noninfected for infected hemocytes.

SUMMARY

1. Although a comprehensive model of baculovirus antigenic relationships is not yet available, it is clear from the presence of BP-gs in numerous nuclear polyhedrosis (and granulosis) viruses that these viruses cannot be classified by host phylogeny.

2. Appropriate controls for anti-insect and/or insect nonbaculovirus predators are negative.

3. We have identified a BP-gs antigen in 15 NPV and two GV polyhedrins. Its concentration is, but its antigenicity is not, dependent on carbonate concentration or dissolution time within the limits discussed (i.e., the BP-gs determinant) and appears to be a repetitive polyhedrin structure independent of the unit size of the polyhedrin employed as assay antigen or immunogen.

4. We have identified four *L. dispar* type-specific (B-Ld-ts) antigens. Their concentration and possibly their antigenic structure are dependent on carbonate concentration, dissolution time, and alkaline protease activity. The origin of these type-specific antigens (polyhedrin vs. virion) has not been clarified. However, the appearance of these B-Ld-ts antigens in polyhedrins released during longer dissolution times leads us to believe they are of virion origin.

5. Immunoelectrophoresis and immunoadsorption electrophoresis assays clearly provide for the possibility of separating the B-Ld-ts and other NPV type-specific antigens for the production of monospecific reagents.

6. Crossed immunoelectrophoresis and immunoelectrophoresis assays indicate that there are several precipitogens in *L. dispar* polyhedrin whose type-specific antigens *cannot* be resolved by the immunodiffusion, complement fixation, and fluorescent antibody assays used for verification of the BP-gs antigen.

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DISCUSSION

COLLINS: I am unclear as to what you think the derivation of the types of the specific antigens are. Are you saying that these are proteolytic cleavage products of a molecule that has a group determinant, or is there any relation? Have you tried to process your polyhedrin material in the presence of protease inhibitors and use this as your immunogen?

DICAPUA: No.

COLLINS: Also, this may be premature, but has anyone looked into those situations in which one finds immunity in animals? Suppose they are in the same area as some of these insects in which it has been recorded that a certain human population in Malaysia has antibodies against some of the baculoviruses. Is that immunity group specific or type specific?

HARRAP: We have approached this problem in another way. We have tried to separate the components of the viruses first, making antisera independently of polyhedrin, enveloped nucleocapsids, and nucleocapsids. We found, using the Spodoptera model, that antisera to enveloped virus particles have a number of common or cross-reacting antigens in immunodiffusion, but one or maybe two specific ones. When we examined nucleocapsid antisera, we could discriminate between two of the viruses. The antiserum to the third was lost. Certainly two of them have a specific immunodiffusion line. This was surprising because we expected type specific responses to be on the envelope.

Now both these antisera will specifically recognize virus infection in cell culture with the appropriate virus, and you can detect viral antigen by 20-24 hours post-infection in individually infected cells. There is no cross reactivity using the nucleocapsid antiserum. In fact, in immunofluorescence, cross reactivity with the virus particle's antiserum is very slight. Using immunodiffusion, we can detect cross reaction.

COLLINS: I would not be too surprised about the type specificity in capsid proteins. There is a lot of precedence in the C-type viruses which have type-specific determinants, both on glycoproteins and envelope major capsid protein.

IGNOFFO: How sensitive is the HA assay?

DICAPUA: We can detect about 50 mg material; it is not very sensitive in terms of HA technique.

Recent Advances in Baculovirus Serology: Radioimmunoassay and Immunoperoxidase Assay

Pepper Hoops and Max D. Summers, Ph.D.
Texas A&M University
College Station, Texas

INTRODUCTION

Although baculovirus serology has become an area of expanding interest in recent years, little progress has been achieved in delineating the species-specific relationships desirable for the development of immunological identification and detection techniques capable of evaluating viral interactions in biological systems. Furthermore, many of the results of past serological investigations are difficult to interpret because of a variety of purification procedures that have been utilized for the preparation of antigens for antisera production. Along with this, few workers have documented the purity and stability of antigen preparations through biophysical and biochemical methods. Also, highly sensitive and quantitative serological techniques presently used for the antigenic characterization of viruses and detection of viral activity with vertebrate and plant viruses have not been routinely applied in baculovirus serological research.

ANTIGEN PREPARATION AND EVALUATION

Advances in the biochemical characterization of baculoviruses emphasize the necessity for employment of antigen preparations of known purity and stability. Recent reports have documented the presence of an alkaline activated protease associated with granulosis and nuclear polyhedrosis viruses, which under normal isolation procedures routinely used by insect virologists, is capable of the degradation of polyhedrin and granulin (1, 2, 3). Antigen heterogeneity, as a result of enzyme degradation and the effect upon subsequent serological evaluation, can be demonstrated with immunodiffusion studies.

Figure 1 illustrates the immunodiffusion patterns obtained when an antiserum, raised against *Trichoplusia ni* (TnGV) granulin (26,000 mol. wt.), isolated under conditions designed to inhibit protease activity, and subsequently further purified by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (3), was used to compare the antigenic relationships of eight baculovirus polyhedrins and granulins. *Trichoplusia ni* granulin forms three precipitin lines with homologous antiserum. *Spodoptera frugiperda* (SfGV) granulin, structurally very similar as determined by two-dimensional peptide mapping (4), shows a reaction of identity with two of the precipitin lines (Figure 1A). *Autographa californica* (AcMNPV), *Rachiplusia ou* (RoMNPV), *Heliothis armigera* (HaMNPV), *Anticarsa gemmatalis* (AgMNPV), and *Trichoplusia ni* (TnSNPV) polyhedrins demonstrate reactions of identity with two precipitin lines (Figure 1B, C). *Heliothis zea* (HzSNPV) polyhedrin shows a reaction of identity with one precipitin line (Figure 1C). However, the TnGV granulin antiserum also recognized additional antigenic determinants on AcMNPV, RoMNPV, HaMNPV, AgMNPV, and TnSNPV polyhedrins reflected as either nonidentity or partial identity reactions (Figure 1B, C).

When the same antiserum was reacted with eight polyhedrins and granulins demonstrating considerable degradation believed to be induced by enzyme activity, the diffusion patterns in Figure 2 resulted. The reaction of identity seen in Figure 1A between TnGV and SfGV granulins now appears as a reaction of partial identity, as indicated by spur formation. Also, the location of the cross-reacting precipitin lines has shifted toward the antigen well. The reactions of identity between TnGV granulin and AcMNPV, RoMNPV, HaMNPV, AgMNPV, HzSNPV, and TnSNPV polyhedrins observed in Figures 1B and C can no longer be detected. Again, only partial identity reactions appear for AcMNPV, RoMNPV, HaMNPV, and TnSNPV polyhedrins. Degraded AgMNPV and HzSNPV polyhedrins do not react with this antiserum.

Thus, the ability of TnGV granulin antiserum to discern serological relationships among these proteins has been altered as a result of the physical integrity of the antigen preparations used. Past attempts at serological characterizations of polyhedrins and granulins may have suffered from similar artifacts. Therefore, efforts involving production of antisera

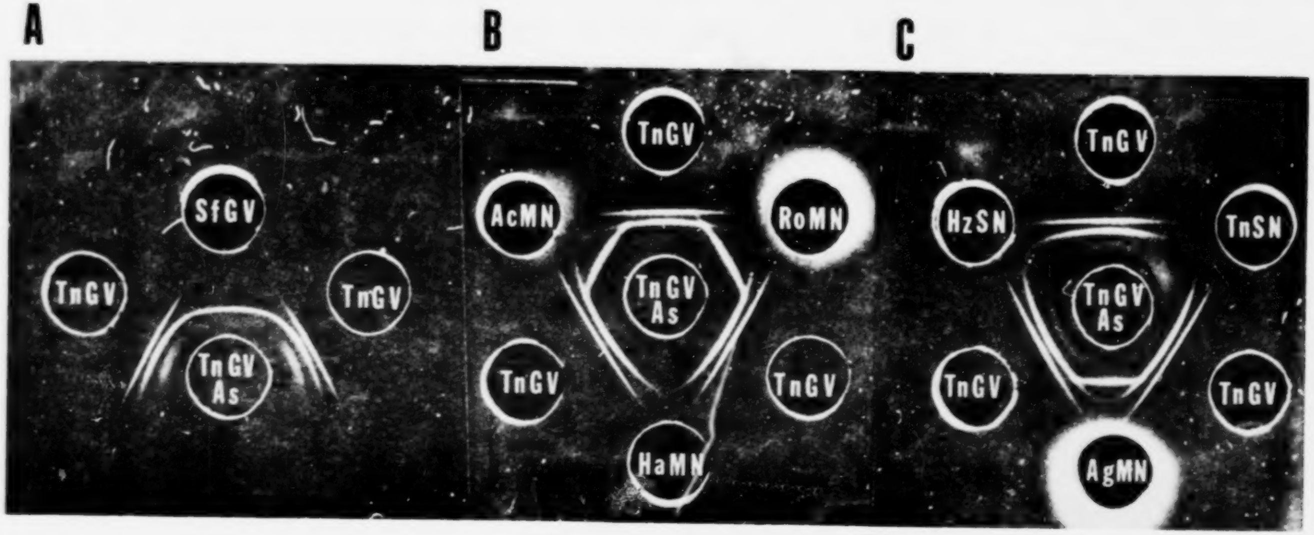
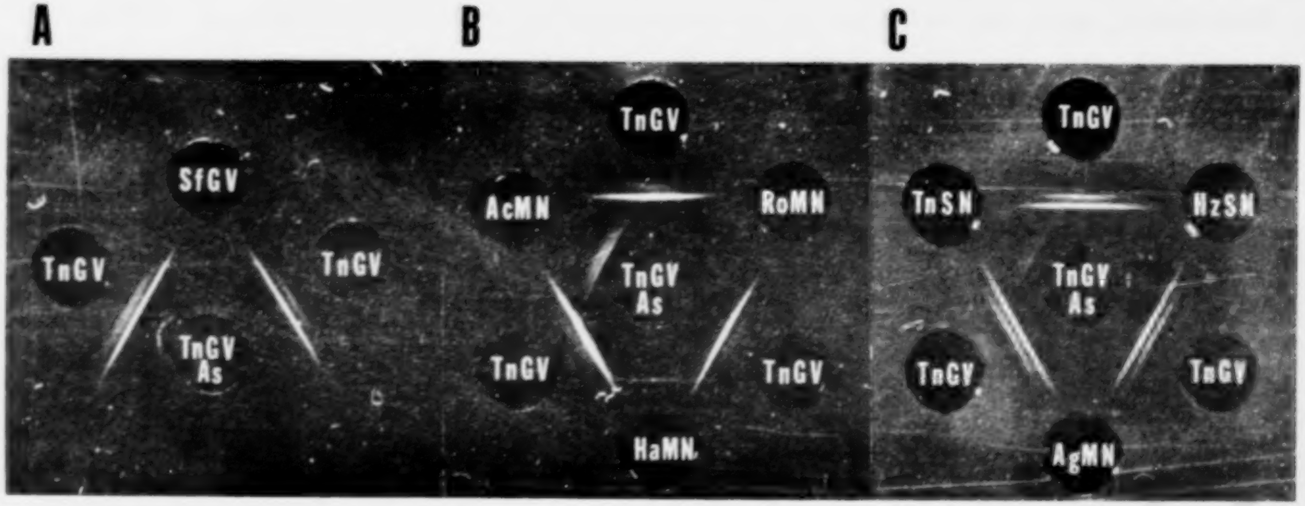

A
B
C
TnGV
SfGV
TnGV
TnGV As
AcMN
TnGV
RoMN
TnGV As
TnGV
TnGV
HaMN
HzSN
TnGV
TnSN
TnGV As
TnGV
TnGV
AgMN

Figure 1. Immunodiffusion of purified polyhedrins and granulins using *Trichoplusia ni* granulin antiserum (TnGV As). (A) Comparison of *Trichoplusia ni* (TnGV) and *Spodoptera frugiperda* (SfGV) granulins; (B) Comparison of *Trichoplusia ni* (TnGV) granulin with *Autographa californica* MNPV (AcMN), *Rachiplusia ou* MNPV (RoMN), and *Heliothis armigera* MNPV (HaMN) polyhedrins; (C) Comparison of *Trichoplusia ni* (TnGV) granulin with *Anticarsa gemmatalis* MNPV (AgMN), *Trichoplusia ni* SNPV (TnSN), and *Heliothis zea* SNPV (HzSN).


A
B
C
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AcMN
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RoMN
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TnGV
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HaMN
TnSN
TnGV
HzSN
TnGV As
TnGV
TnGV
AgMN

Figure 2. Immunodiffusion of enzyme degraded polyhedrins and granulins using Trichoplusia ni granulin antiserum (TnGV As). Abbreviations for granulins and polyhedrins tested are the same as in the legend for Figure 1.

with discriminating properties must be related to standardized antigen preparations routinely monitored for purity and stability.

RADIOIMMUNOASSAY

Many of the standard serological methodologies have been employed in the antigenic characterization of baculoviruses, including immunodiffusion (5-15), immunoelectrophoresis (16, 17), immunofluorescence (18-20), complement fixation (21-26), hemagglutination (27-30), and viral neutralization (20, 31). However, improved serological techniques widely used in other virological systems have not been routinely applied to baculovirus studies.

Radioimmunoassay (RIA), the most sensitive immunological assay for quantitating biological substances, has just recently been applied to the measurement of baculovirus antigens. Kalmakoff et al. (32) report a radioimmunoassay for polyhedrin of Wiseana cervinata which can detect 3×10^4 polyhedra per ml, approximately 1 μg protein. Furthermore, this assay can distinguish W. cervinata polyhedrin from B. mori NPV polyhedrin.

Realizing that more quantitative applications utilizing serological techniques with both improved sensitivity and specificity are desirable, the following preliminary data on the application of radioimmunoassay (RIA), immunoradiometric assay (IMRA), and immunoperoxidase are presented.

A microtiter solid-phase RIA and IMRA based upon the assay for hepatitis B viral antigens described by Purcell et al. (33) is being investigated in our laboratory for the detection and antigenic comparison of baculovirus granulins, polyhedrins, and enveloped nucleocapsids. Figure 3 shows the ability of log dilutions of AcMNPV polyhedrin ranging from 10 pg to 10 μg to compete with ^{125}I-AcMNPV polyhedrin for antibody binding. Using 10% displacement as the lowest limit of significant detection, this assay can detect 75-100 pg of homologous antigen. This represents approximately 40 polyhedra. Immunoradiometric assay, a modification of RIA employing ^{125}I-labeled AcMNPV polyhedrin antibodies, displays a similar sensitivity as indicated in Figure 4. Of particular note in this assay is the specificity of binding of AcMNPV polyhedrin antiserum compared to homologous viruses. Neither AcMNPV LOVAL (Larval derived Occluded Virus that has been Alkali Liberated) nor PMB-NOV (Plasma Membrane Budded Nonoccluded Virus) shows binding of ^{125}I-AcMNPV polyhedrin antiserum at the sensitive levels of detection displayed in this experiment, i.e., below 200 ng antigen concentration.

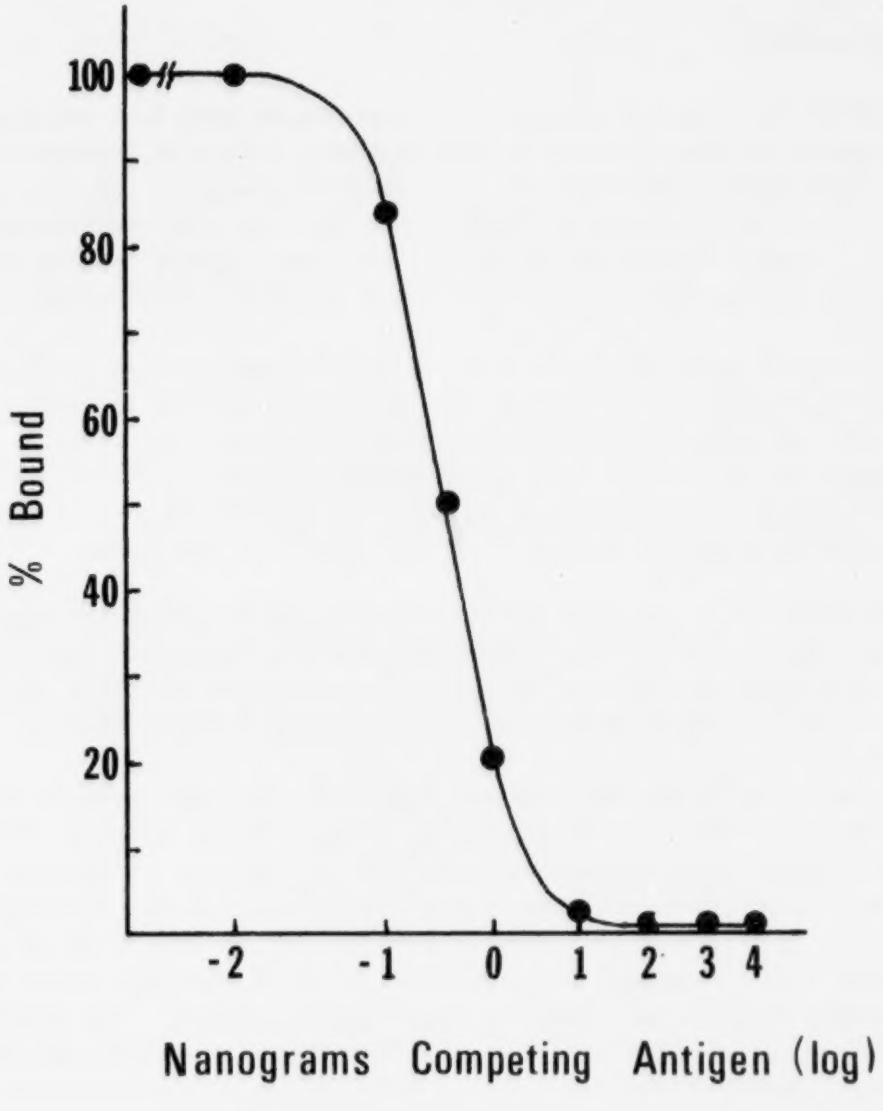


Figure 3. Radioimmunoassay of AcMNPV polyhedrin. The assay measured AcMNPV polyhedrin (●) by the ability of unlabeled polyhedrin to compete with a limiting dilution of AcMNPV polyhedrin antiserum for binding ^{125}I-AcMNPV polyhedrin. The results are expressed as the total bound ^{125}I-polyhedrin standardized to 100% in the absence of competing antigen.

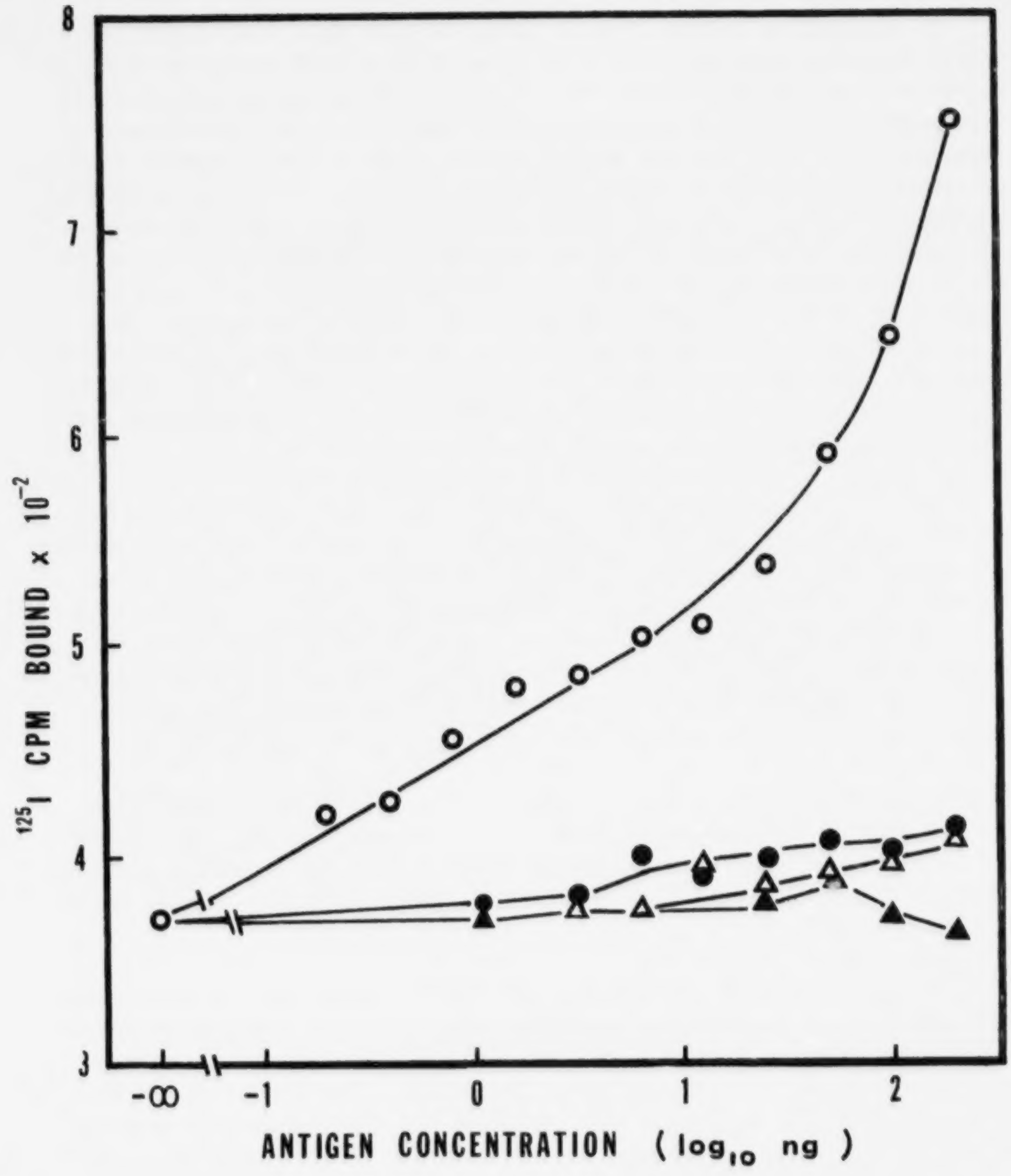


Figure 4. Immunoradiometric assay of AcMNPV polyhedrin. Log dilutions AcMNPV polyhedrin (o), AcMNPV LOVAL (•), and AcMNPV PMB-NOV (Δ) were incubated in an excess of AcMNPV polyhedrin antiserum and measured by the ability of bound antigen to bind ^{125}I-IgG from polyhedrin antiserum. Preimmune serum binding of AcMNPV polyhedrin is indicated by (▲).

In comparative immunodiffusion tests, we have found that AcMNPV polyhedrin antiserum does not react with either SfGV or TnGV granulins at equivalent antigen concentrations. When SfGV and TnGV granulins were compared with AcMNPV polyhedrin by competitive RIA, the results in Figure 5 were obtained. Both SfGV and TnGV granulins were shown by RIA to possess similar antigenic determinants to AcMNPV polyhedrin, as evidenced by the ability to compete for antibody binding. However, while 0.5 ng of AcMNPV polyhedrin was necessary to displace 50% of the homologous ^{125}I-AcMNPV polyhedrin, 20 and 25 ng representing 40- and 50-fold increases in relative antigen concentrations of TnGV and SfGV were required for equal displacement. Upon utilization of appropriate antigen ratios, cross reactions of TnGV and SfGV granulins with AcMNPV polyhedrin have been recently confirmed in immunodiffusion tests. In this comparison, the advantages of a more sensitive and quantitative immunological assay are evident.

In order to test the RIA for the detection of polyhedrin *in vivo* and thereby evaluate polyhedrin antisera against a naturally produced polyhedrin, the measurement of polyhedrin in AcMNPV infected TN-368 cell cultures was undertaken. Initial aliquots of 10^6 cells which had been infected for 18 hours were disrupted, and 10-fold dilutions of the cell supernatants were tested by RIA. Based upon a standard curve of uninfected cells with known concentrations of purified AcMNPV polyhedrin added, polyhedrin could be detected in as few as 100 cells with a sensitivity of 1 ng polyhedrin.

A kinetic experiment was then performed to ascertain when polyhedrin synthesis could first be detected during the infection cycle. The results indicate that polyhedrin synthesis can first be detected between 8 and 12 hours post-infection, which correlates with immunoperoxidase studies to be described later.

Competitive RIA of polyhedrins and granulins from eight baculoviruses have now been conducted utilizing AcMNPV polyhedrin and TnGV granulin antisera. The numerous cross reactions seen in immunodiffusion studies have been confirmed and furthermore quantified by RIA. The results indicate that many antisera would have to be prepared and screened to find possible discriminating reagents for the identification of baculoviruses using polyhedrin and granulin as a standard.

The detection of enveloped nucleocapsids has also been evaluated by RIA. In a competitive RIA of purified enveloped nucleocapsids from six nuclear polyhedrosis and two granulosis viruses using an ^{125}I-AcMNPV-anti-AcMNPV

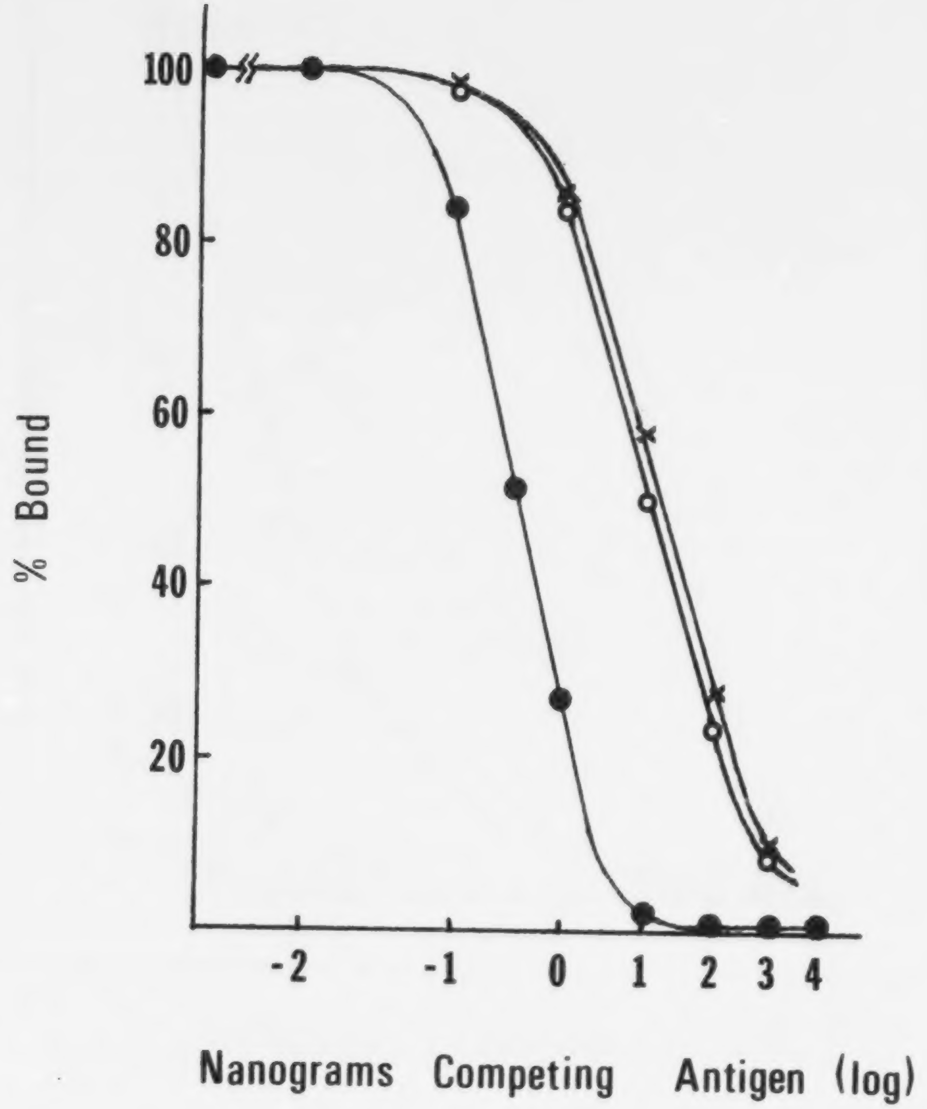


Figure 5. Immunological comparison of AcMNPV polyhedrin, SfGV, and TnGV granulins by radioimmunoassay. The assay measured the ability of unlabeled antigen to compete with a limiting dilution of AcMNPV polyhedrin antiserum for ^{125}I-AcMNPV polyhedrin binding. (•) AcMNPV polyhedrin; (o) SfGV granulin; and (X) TnGV granulin.

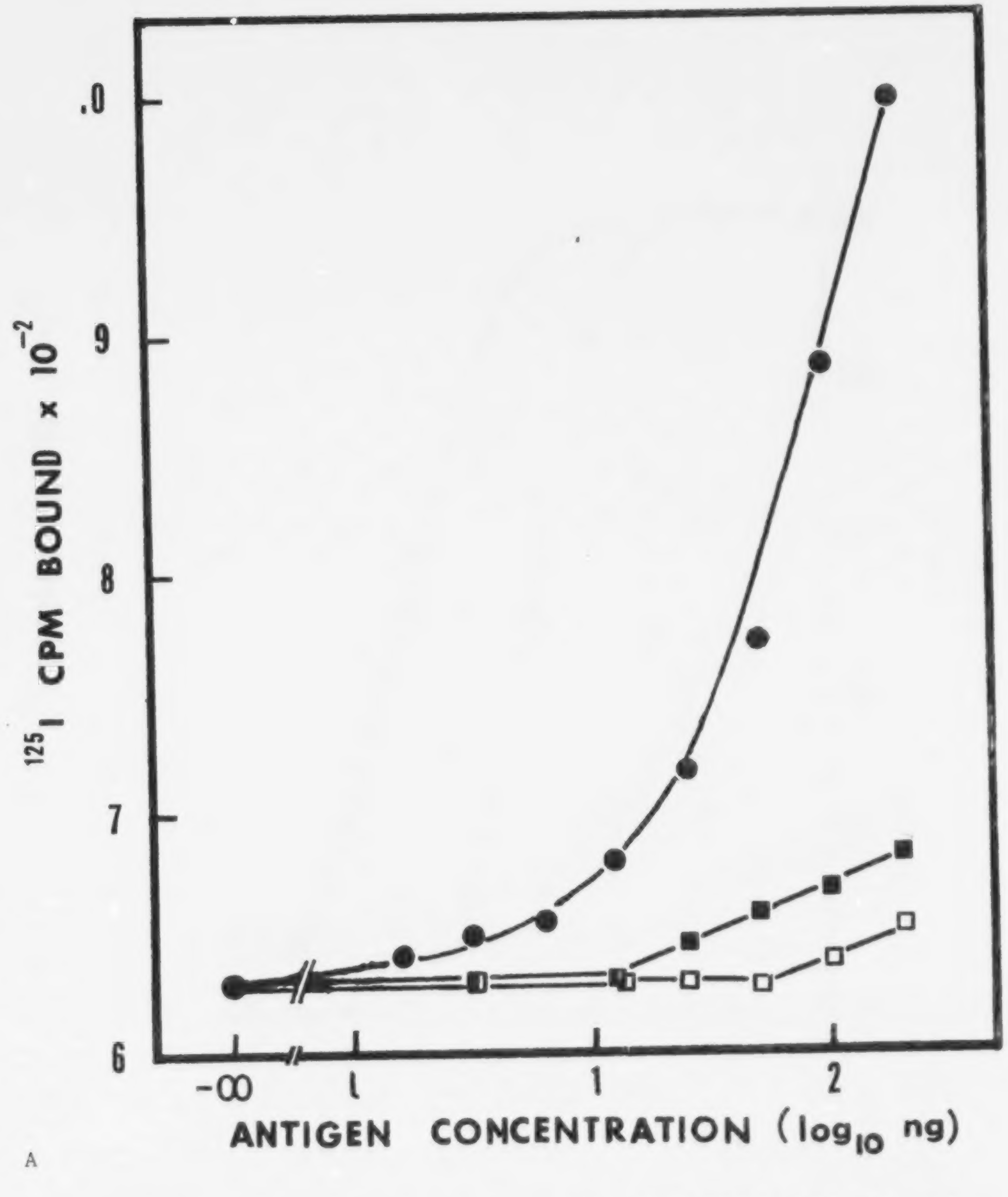
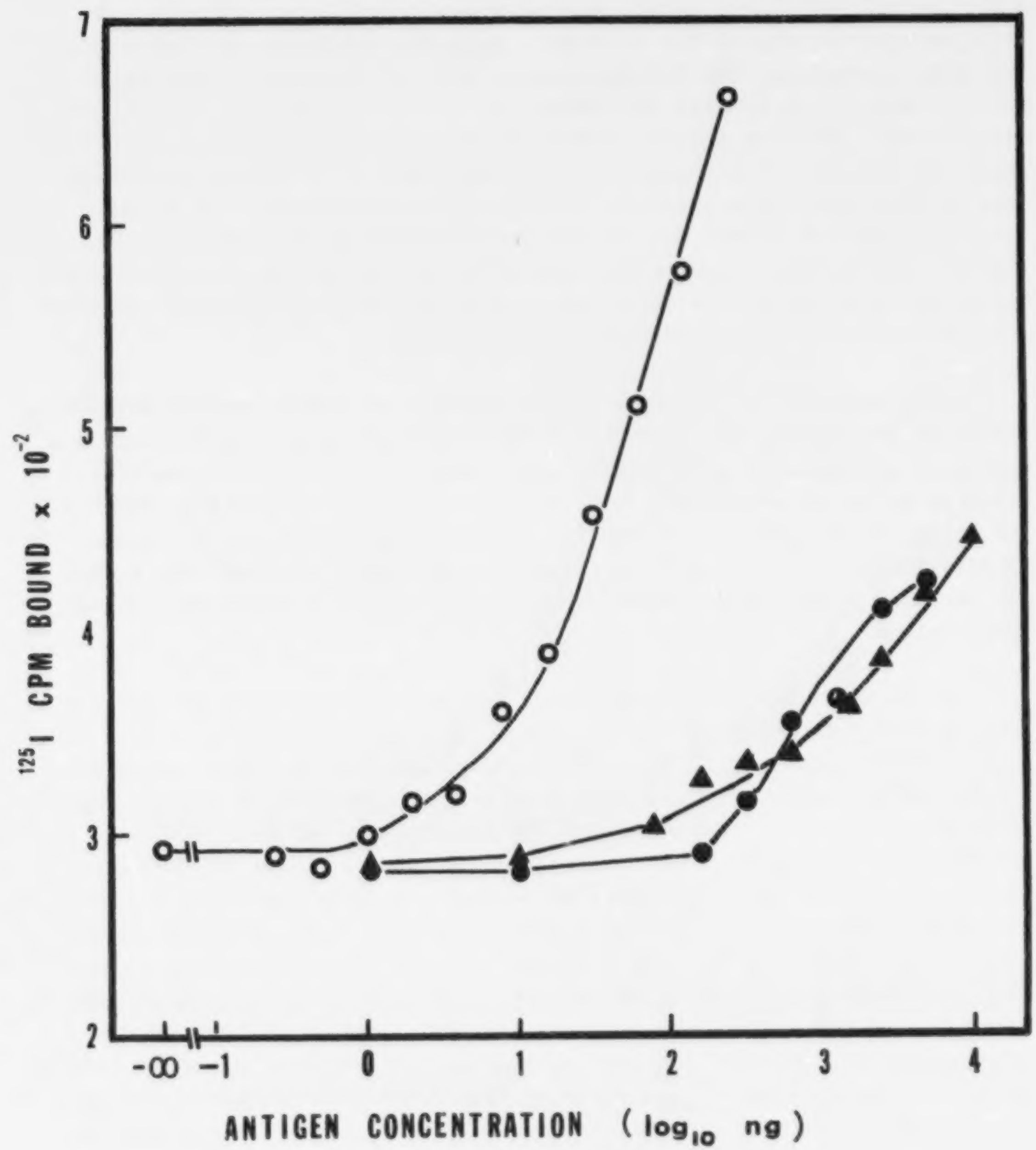


Figure 6. Immunological comparison of AcMNPV LOVAL, PMB-NOV, and polyhedrin by immunoradiometric assay as described in Figure 4. (A) AcMNPV LOVAL antiserum and ^{125}I-IgG binding were employed to measure AcMNPV LOVAL (●), AcMNPV PMB-NOV (■), and AcMNPV polyhedrin (□). (B) AcMNPV PMB-NOV antiserum and ^{125}I-IgG were used to measure AcMNPV PMB-NOV (○), AcMNPV LOVAL (▲), and AcMNPV polyhedrin (●).



^{125}I CPM BOUND x 10^{-2}
7
6
5
4
3
2
-∞
-1
0
1
2
3
4
ANTIGEN CONCENTRATION ($\log_{10}$ ng)

Figure 6B

assay system, AcMNPV and RoMNPV demonstrate no significant difference in their ability to compete for antibody. Heliothis armigera, Heliothis zea, Anticarsa gemmatalis, and Trichoplusia ni NPVs and Spodoptera frugiperda and Trichoplusia ni GVs did not compete at 10,000-fold greater antigen concentrations. Antisera against RoMNPV purified enveloped nucleocapsids also could not distinguish homologous virus from AcMNPV in a similar experiment. Thus, AcMNPV and RoMNPV antisera recognize common antigenic determinants between AcMNPV and RoMNPV not present or accessible in the other viruses tested. Furthermore, comparative neutralization studies demonstrated AcMNPV antiserum could neutralize these two viruses with equal efficiency, supporting the close relatedness of these viruses (31).

Polyacrylamide gel analysis in the presence of sodium dodecyl sulfate indicates that AcMNPV and RoMNPV purified enveloped nucleocapsids contain a number of polypeptides with similar electrophoretic mobilities. However, proteins or unique mobilities for each virus were also observed by Summers and Smith, whose findings are now in press. Therefore, the production of identifying antisera for closely related viruses such as AcMNPV and RoMNPV may ultimately require isolation of unique structural proteins for use in immunization.

Immunoradiometric assays were also performed to determine whether the multiple viral forms characteristic of many baculovirus infections were serologically distinguishable (31). ^{125}I-labeled antisera to AcMNPV LOVAL and AcMNPV PMB-NOV were used in binding studies with each form of virus. Figure 6A shows that AcMNPV LOVAL antiserum can discriminate between LOVAL and PMB-NOV forms of AcMNPV. The reciprocal binding assay using PMB-NOV antiserum gave similar results (Figure 6B). However, cross reactions did occur with heterologous forms at higher antigen concentrations. Neutralization studies conducted with the same antisera indicate that while PMB-NOV antisera will neutralize LOVAL, LOVAL antiserum did not neutralize the PMB-NOV form under the experimental conditions employed. This reveals a complex serological relationship from the standpoint of neutralization antigens (31). Again, the identification of viruses at this level will require either more discriminating antisera or antisera raised against isolated virus-specific antigens.

IMMUNOPEROXIDASE

Plaque formation, which is the result of polyhedra formation and currently the routine method for measurement of virus infectivity *in vitro*, is now being questioned in light of increasing evidence of host or tissue cell influence upon the synthesis and assembly of this protein. Immunological techniques employing specific viral antigens offer alternative means for the detection of viral activity. To this end, immunoperoxidase has been employed in screening infected cells for the detection of enveloped nucleocapsid replication relative to polyhedrin synthesis and polyhedra production.

When TN-368-10 cell lines were infected with AcMNPV and monitored for the presence of viral antigens with respect to time using AcMNPV enveloped nucleocapsid and polyhedrin antisera, the typical brown coloration observed in positive reactions appeared at 8-10 hours post-infection with AcMNPV enveloped nucleocapsid antiserum. This corresponds to the appearance of intracellular and extracellular infectious virus as assayed by the conventional polyhedral plaque assay (34, 35). The substitution of preimmune serum for immune serum abrogated the reaction, indicating the specificity of the immunoperoxidase assay. AcMNPV polyhedrin antiserum first detected polyhedrin at 12 hours post-infection, preceding detectable polyhedra formation by 2 hours. Again, preimmune sera showed no reaction.

The infection of several other insect cell lines with AcMNPV at the level of polyhedrin synthesis and enveloped nucleocapsid replication were also monitored using immunoperoxidase. Significantly, it was found that *Bombyx mori* cells were infected with AcMNPV as indicated by the positive reactions obtained with AcMNPV enveloped nucleocapsid antiserum. However, AcMNPV polyhedrin antiserum gave negative reactions. Infection of *B. mori* cells has been confirmed in supporting studies through back titration of infected cell supernatants in TN-368-10 cell lines, yielding titers of 10^6 PFU/ml. Thus, the presence of polyhedrin, and as a result polyhedra formation, may not be an indication of infection by baculoviruses in new systems that have not been characterized. This confirms the need to develop detection techniques specific for enveloped nucleocapsid replication in addition to polyhedra production.

SUMMARY

Relative to the results of early and even more recent reports on the serology of baculoviruses, some of which are difficult to interpret, it is obvious that one of the major problems was, and is, that of antigen purity and specific criteria utilized to establish such. It is easily observed that techniques are now available to routinely monitor and identify the composition of baculoviruses and prepare the basis for developing reproducible and standardized antigens as reagents. Until we are more confident of the sensitivity and specificity of our serological techniques and results, it will be important to reference those results relative to, for example, SDS-PAGE profiles of virus and/or viral proteins.

The example of the apparent group-related baculovirus antigens of granulins and polyhedrin sets the precedent for this observation. Individual baculoviruses have been shown to have a unique polyhedrin or granulin associated with them. However, these proteins have also been shown to have similar or related primary structures by two-dimensional peptide mapping (4). The serological data presented confirm this. Based upon what we know about the structure and chemical and physical properties of this class of proteins, it should be theoretically possible to develop agent-specific antisera to each one. However, we have to consider the need for it because of the potential problems of relying on the presence of polyhedrin or granulin as a reliable indicator of infection.

It seems more reasonable to concentrate more attention toward the serological and structural characterization and comparisons of baculovirus enveloped nucleocapsids. However, the complex structure of these viruses reveals that this task may not be an easy one. Within the virion we need to probe for group-related and virus-specific proteins and develop antisera accordingly.

The preliminary results, herein with a limited array of baculovirus antisera which are likely not of desirable RIA quality, appear promising. As in vertebrate and plant virology, serological probes can be developed for kinetic studies which are both specific and sensitive to record the fate of baculoviruses and baculovirus proteins. Once the appropriate proteins have been identified, highly specific antisera can be developed against purified viral antigens.

The area of baculovirus serology and immunology does have historical precedence, but relative to the present discussion, knowledge of baculovirus structure and activity in cellular systems, baculovirus serology is entering a new dimension of use and application. It is not likely going to be an easy area of investigation because of the structural and genetic complexity of baculoviruses. At the moment we must focus our attention toward simple, yet reliable techniques for identification, but most of all, techniques must be used which work, regardless of simplicity. We must refine serological techniques to the ultimate obtainable in terms of detection and sensitivity relative to retaining the specificity of our antisera. Finally, from this we will develop routine, standardized procedures and reagents which are more reliable than is presently the situation.

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DISCUSSION

HEIMPEL: What are your sources of viruses? Do you get them from one source, or is a conglomerate of resources combined to get them?

HOOPS: Which viruses?

HEIMPEL: For example, the Rachiplusia ou and the Autographa californica viruses. Do you have one source or do you obtain them from many sources?

HOOPS: Our source of Rachiplusia ou is from Dr. Kawanishi. We have various sources of Autographa californica, but we used just one source for these studies.

GRANADOS: How can you explain the apparent absence of the Autographa virus from the nucleus of Bombyx mori cells?

HOOPS: If you will remember, by using Autographa californica NPV antiserum, you saw a reaction in Bombyx mori cells. It appeared predominantly in the cytoplasm. This, we think, points to the fact that even though nucleocapsid assembly occurs in the nucleus, our antisera recognize predominantly envelope nucleocapsid antigen associated with those particles that are occluded. So, the large amount of staining is not visible that is normally seen in infected TN-368 cells, where an assembly occurs of envelope nucleocapsids in the nucleus.

GRANADOS: This is good evidence then that the envelope nucleocapsid within the nucleoplasm itself is distinct, and that it acquires the membrane during the budding through the nuclear membrane.

HOOPS: We do not have enough information to make that kind of statement based upon present evidence.

GRANADOS: Your assay does not detect envelope virions in the *B. mori* nucleus, and yet, we know there is envelope material.

HOOPS: Previous studies have shown considerable numbers of nucleocapsids in the virogenic stroma. We know that in most systems the envelope material is assembled in the nucleus; however, we do not yet know that for *Autographa californica* in *Bombyx mori*.

Cell Culture Studies: Standardization of Biological Activity

Loy E. Volkman, Ph.D.
Lovelace-Bataan Medical Center
Albuquerque, New Mexico

In considering the topic of standardization of biological activity in cell culture, I would like to discuss some aspects of the nature of both the host lepidopteran cells and the parasitic nuclear polyhedrosis viruses, since the recognition of infection is based on some change due to the interaction of both components of the host/parasite system. I would like to examine the basis of the assays that are now used for determining MNPV biological activity *in vitro* and for quantitating biological activity, and discuss possible risks involved in relying on these assays.

First, I'll briefly review some characteristics of the MNPVs as they behave in cell culture (M refers to the multiple-nucleocapsids-per-envelope type of NPV). So far as I know, attempts to replicate SNPVs (single-nucleocapsids-per-envelope) and granulosis viruses in cell culture have been unsuccessful.

Figure 1 is a schematic diagram of the many different forms that can occur in an MNPV infected TN-368 cell. The occluded form, of course, occurs in the nucleus, but there also are nonoccluded forms in the nucleus, only some of which are destined to become occluded. What fundamental factors are involved in determining which nucleocapsids become occluded and which do not are not known at this time, but it does seem that being enveloped is a prerequisite for nucleocapsid occlusion (1). There are

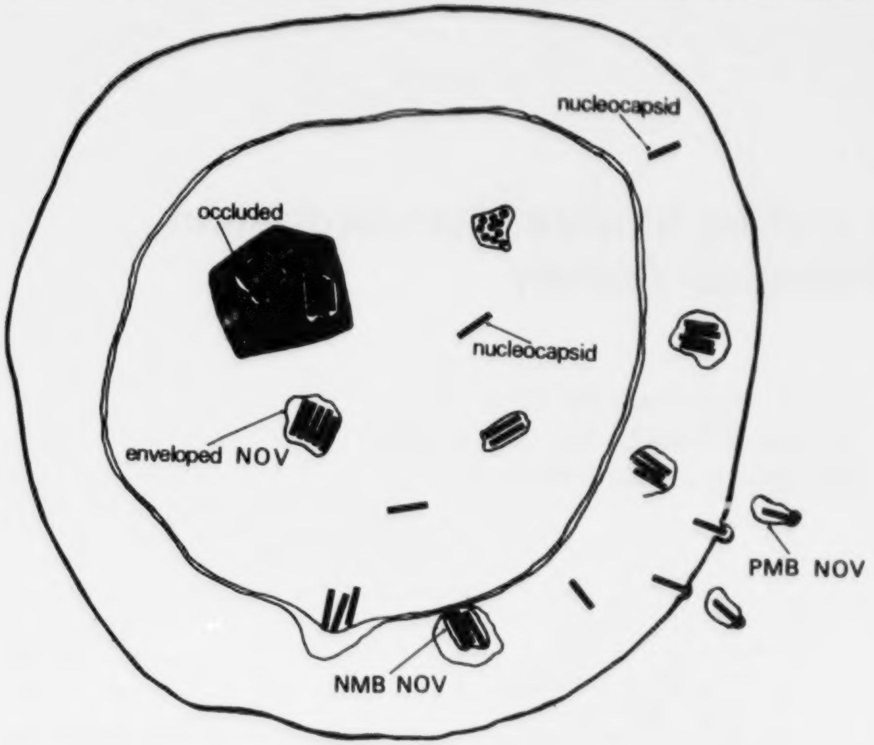

Figure 1. A schematic diagram illustrating the many different phenotypic forms of virus that occur in an Autographa californica MNPV infected TN-368 cell.

groups of nucleocapsids as well as single nucleocapsids in the nucleus that are unenveloped. As Hirumi et al. (2), Knudson and Harrap (3), and Mac Kinnon et al. (4) have described, some of these nucleocapsids bud from the inner nuclear membrane into the cisternae of the endoplasmic reticulum. The enveloping membranes do not appear to be stable, however, because frequently nucleocapsids have been seen being released into the cytoplasm through breaks in the membranes. Perhaps this then is at least one of the sources of the single nucleocapsids that bud from the plasma membrane (Figure 2). In the Autographa californica MNPV/TN-368 system, the viruses that bud from the plasma membrane before 48 hours post-infection are primarily in the single-nucleocapsid-per-envelope form with occasional double nucleocapsids per envelope (5). The envelope is very loosely arranged about the nucleocapsid (5). Thus, it is evident that this virus system entails

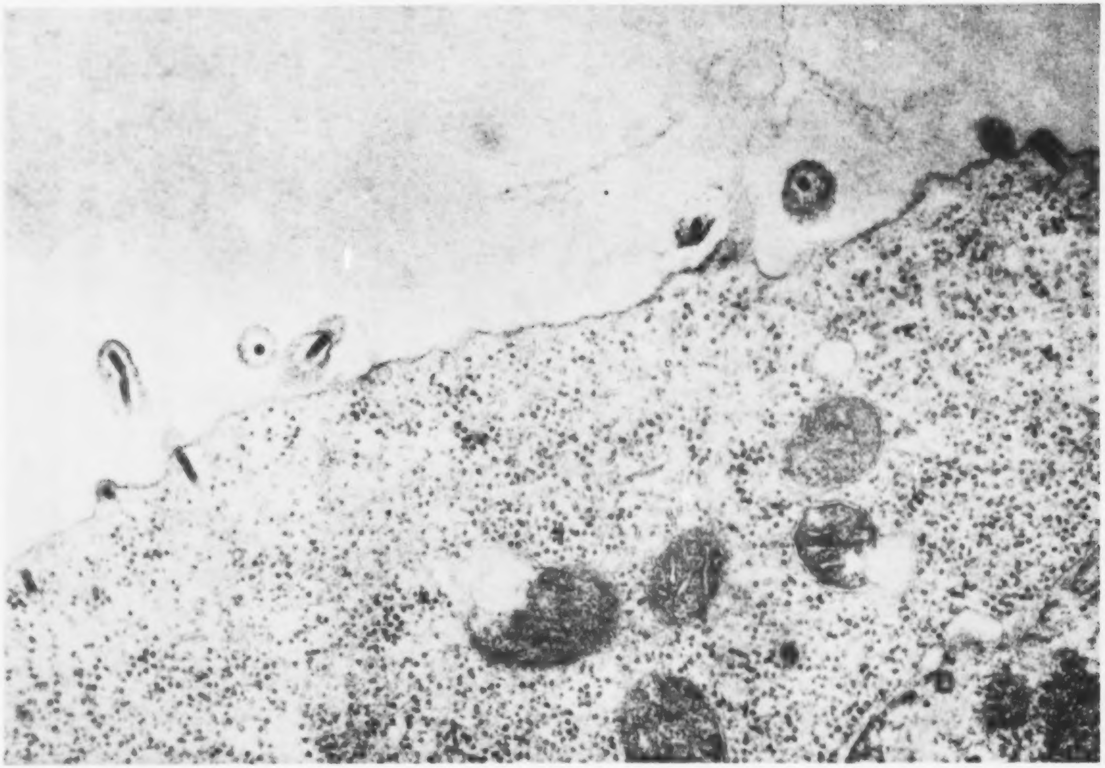

Figure 2. Thin section containing *Autographa californica* MNPV in the process of exocytosis from a TN-368-10 cell.

several variables that should be considered in the standardization of biological activity. The occluded particles are said not to be infectious in vitro until they are released from the polyhedra (6). That still leaves intranuclear unenveloped nucleocapsids, various sizes of intranuclear bundles of nucleocapsids whose envelopes were derived by probable de novo synthesis (7), various sizes of intracytoplasmic bundles of nucleocapsids whose envelopes were derived by budding through the inner nuclear membrane into cisternae of the endoplasmic reticulum, various stages of decomposition of the membranes of the latter, unenveloped intracytoplasmic nucleocapsids, and, finally, what we call a plasma membrane budded nonoccluded virus, which is, again, primarily single nucleocapsid per envelope.

What about the infectivity of all these various forms of particles? Does the source of the envelope, for instance, or the size of the bundle have an effect on the infectivity? It is not unreasonable to expect that the source of the envelope might have a bearing on the host range, or at least the tissue range, and that the number of genome equivalents in the infecting unit might have an effect on the resulting cytopathic effect, or at least the time course of that effect. Another difficulty encountered with this system is in the concept of "multiplicity of infection" because one is dealing with a variable number multicapsid virus. As long as multiple genome equivalents are bundled together as a group, they can meet all the experimental requirements of single hit kinetics (and thus effect a Poisson distribution), and yet it is possible that multiple complements of genomes are required to induce certain types of cytopathic effects. These are possibilities that need to be examined. To do this one needs to purify each of these different forms and determine what its potential is. So far the best characterized form of in vitro produced virus is the plasma membrane budded nonoccluded virus (PMB-NOV). This is so, simply because it is the most easily purified.

Figure 3 demonstrates the effect of infection by MNPV on the multiplication of TN-368 cells. TN-368-13 and TN-368-10 are sublines started from single cells isolated from Fred Hink's parent cell line of Trichoplusia ni virgin moth ovarian cells (TN-368) (8). In this particular experiment, the infected cultures were exposed to the PMB-NOV form of Autographa californica NPV at an MOI of 10 to 20 to assure 100% infection of the cells. It is important to note not only the inhibition of cellular division by the infection, but the absence of significant cytolysis before 48 hours post-infection. This means that virus collected from the cell culture medium prior to 48 hours post-infection approaches a pure preparation of PMB-NOV.

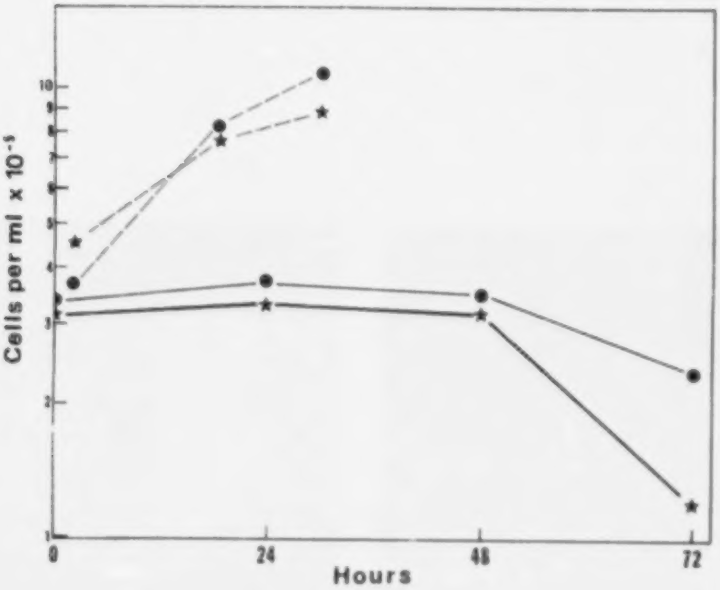

Figure 3. Growth curves of infected (——) and uninfected (---) TN-368-10 (★) and TN-368-13 (●) cells. Cell samples from infected and uninfected cultures were removed periodically and viable cell counts were made. For the infected cultures, the zero hour sample was taken at the end of the adsorption period.

Since PMB-NOV is composed of single enveloped nucleocapsids, "multiplicity of infection" for these particles can be regarded as is standard.

Several things have been learned from studying this form of the virus. We have found that this form can induce polyhedra formation, and when assayed by the polyhedra plaque method on our most sensitive indicator line, TN-368-10, one in 128 particles is infectious (9). We know that in the course of virus production in cell culture, the budding of PMB-NOV precedes the occluding of intranuclear particles, and that as the occlusion process begins, the budding is shut down. This was determined in an experiment in which we attempted to correlate the various morphological stages of cytopathic effect, or CPE, as shown in Figure 4, with a PMB-NOV growth curve. We designated three classes of cytopathic effect: No cytopathic effect, as shown in section A; prepolyhedral cytopathic effect, as shown in B and C (these are early and late, respectively); and polyhedral cytopathic effect, wherein the cells obviously contain one or more occluded virus as shown in D. To do this study we simultaneously infected two TN-368

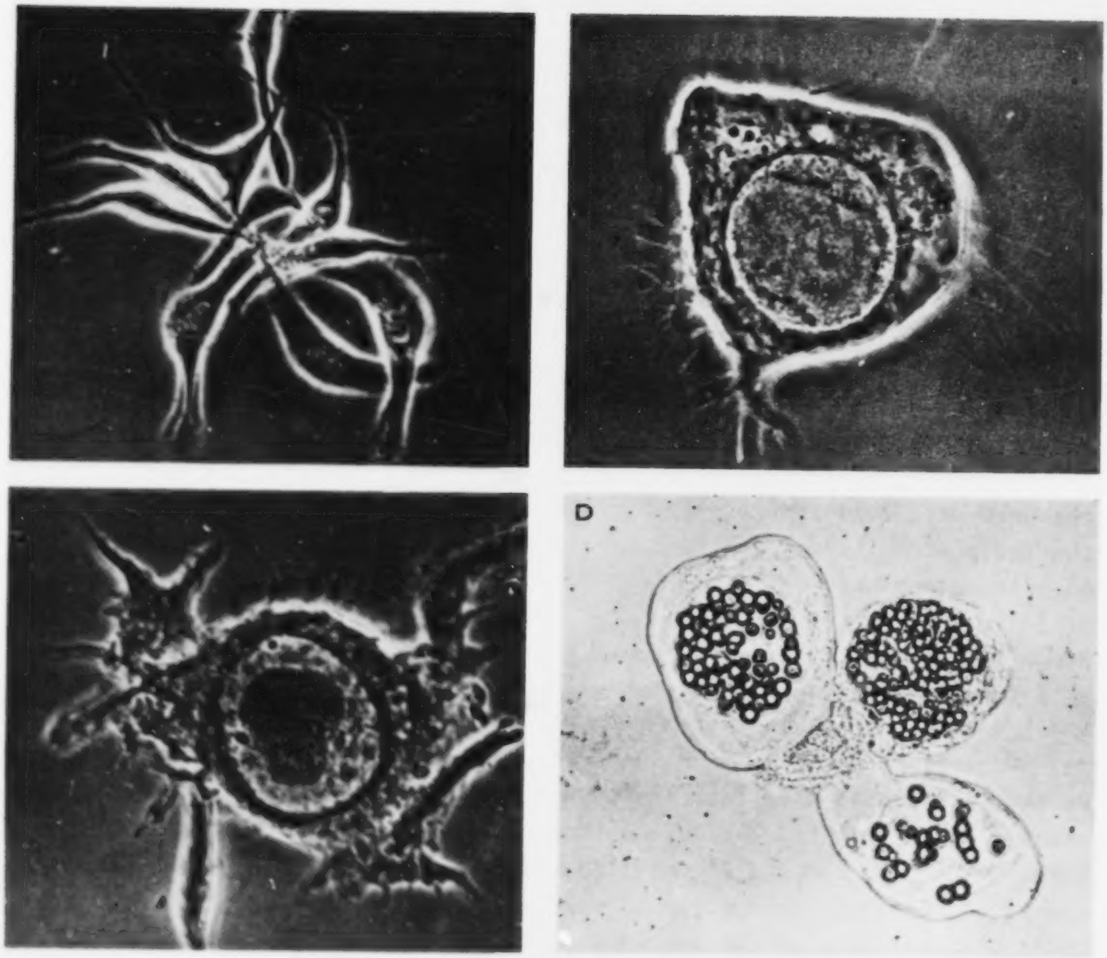

Figure 4. Progression of CPE induced in TN-368 cells by *Autographa californica* MNPV. (A) Uninfected TN-368-13 cells (x3,200). (B) First stage of CPE. The cell has rounded up, the nucleus is enlarged, all nucleoli have disappeared, and the virogenic stroma has formed (x8000). (C) Virogenic stroma seems to have become more compact. Often there is Brownian motion around the internal periphery of the nuclear membrane during this stage (x8000). (D) Cells contain polyhedra (x8000).

sublines, TN-368-10 and TN-368-13, with PMB-NOV at an MOI of 10 to 20, and at various times after infection we removed a sample of the homogeneously suspended cells, titered the supernatant fluid by the plaque assay, and visually inspected at least 300 of the pelleted cells to determine the percentage of cells in each specific stage of cytopathic effect.

Figure 5 shows the results of that study. The open squares represent percent cells showing prepolyhedral cytopathic effect, which begins at just before 10 hours post-infection; the encircled stars represent the percent cells with polyhedral cytopathic effect, which increases, of course, as the prepolyhedral cytopathic effect falls off. The closed circles represent the titer of PMB-NOV in the culture fluid. The virus begins to bud a little before 10 hours post-infection, and continues until about 35 hours post-infection. The histogram represents the change in PFU concentration with time. We see that the budding of the virus corresponds with the prepolyhedral stage of cytopathic effect and that budding is shut down with the onset of polyhedral formation. The results suggest that the budded virus is completed before intranuclear virus becomes occluded.

Further, we know from electron micrographs of preparations of negatively stained gradient purified PMB-NOV in comparison with micrographs of particles that were alkali-liberated from insect grown polyhedra, PMB-NOV have modified surface structures that the latter lack (5). (See Figures 6 and 7A and B.) The infectivity of the isolated single-nucleocapsid-per-envelope alkali-liberated particles from an MNPV preparation is much less than that of PMB-NOV, as assessed by the plaque assay. About 1 in 2.4 x 10^5 particles are infectious, as compared with the PMB-NOV's 1 in 128 (9). Further, there are antigenic differences between the two forms as indicated by their infectivity being neutralized by different populations of antibodies (9). These bits of information indicate that there are significant differences between PMB-NOV and the alkali-released occluded virus. We do not know how the intracellular forms of NOV compare, however. We do know that at least some of the intracellular forms are infectious, but we don't know which ones. It is yet to be definitely established whether or not the unenveloped nucleocapsids are infectious, for example.

The assays most commonly used in evaluating MNPV infectivity in vitro, the plaque assay and the dilution end point assay, use the production of polyhedra as the sign of infectivity (4, 6, 10). The polyhedra produced are not always infectious, however, and they may contain only a few single enveloped nucleocapsids, if any at all. In addition, there may be only 10 or less polyhedra per nucleus. It is this type of polyhedron that appears

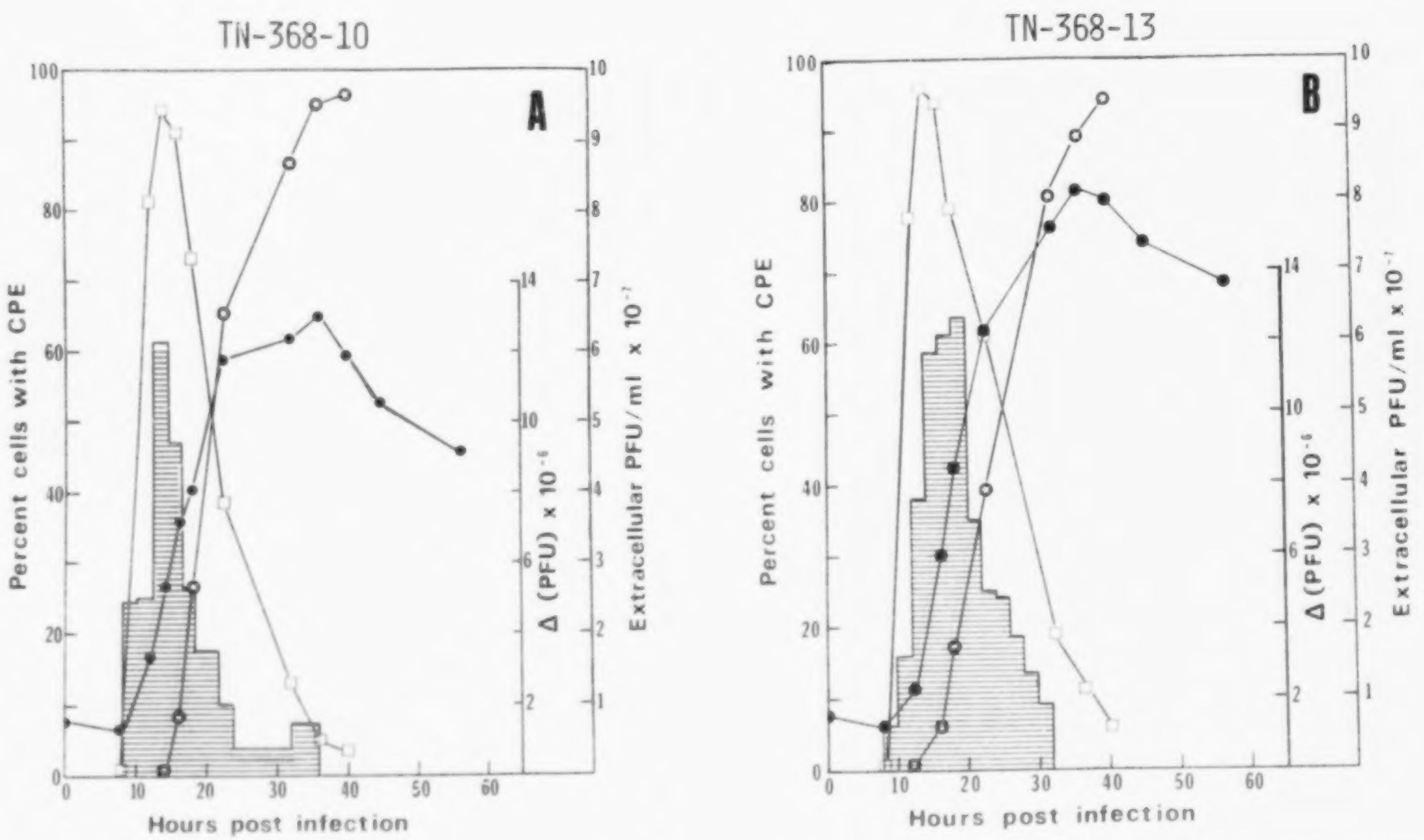

Figure 5. Growth curves of Autographa californica MNPV PMB-NOV in TN-368-10 (A) and TN-368-13 (B) cells correlated with the progressive development of CPE. The appearance of infectious extracellular virus (●) was monitored using a plaque assay, and the development of CPE, both the prepolyhedral stage (□) and the polyhedral stage (◉), was monitored visually by examining 300-400 cells at each sampling with a Zeiss phase microscope. The histogram (▤) represents the change in extracellular virus concentration with time.

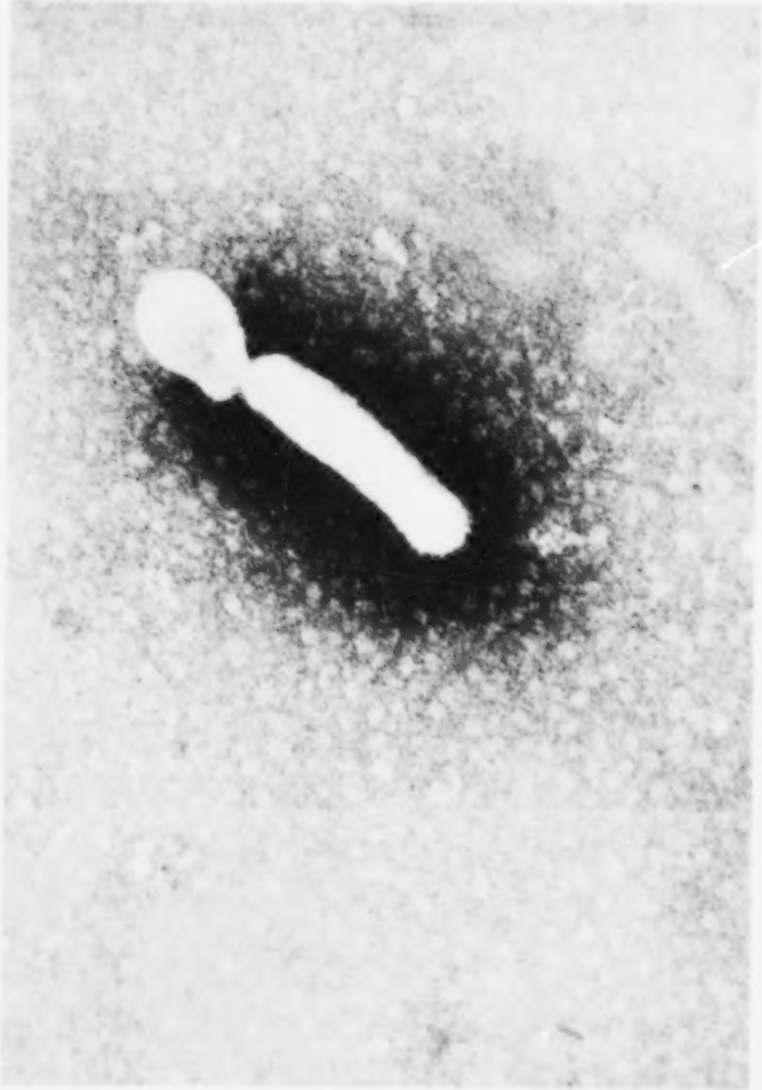

Figure 6. Preparation of Autographa californica PMB-NOV negatively stained with ammonium molybdate, showing the presence of viral envelope in loose and/or variable association and the presence of surface projections or peplomers on the viral envelope (x172,000).

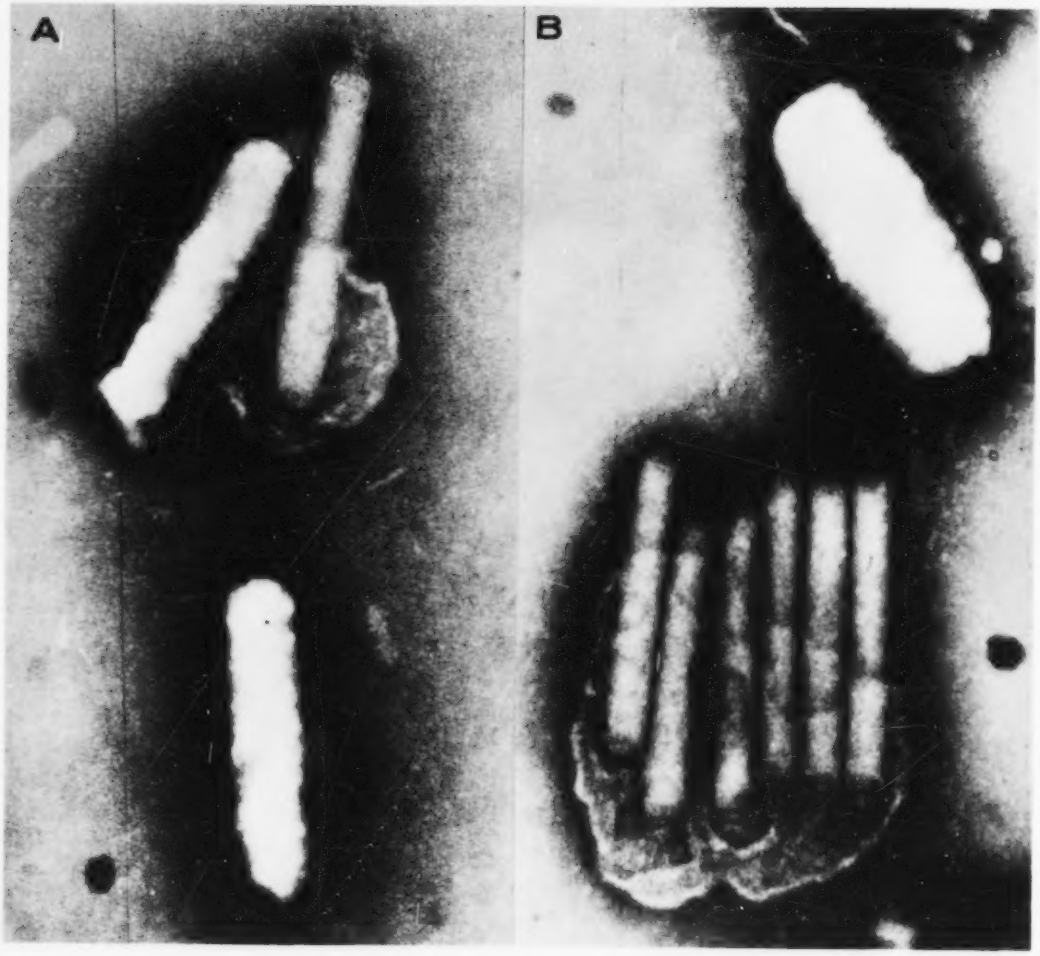

Figure 7. Preparation of alkali-liberated and gradient-purified *Autographa californica* MNPV, negatively stained with ammonium molybdate. (A) Enveloped single nucleocapsids with envelope intact or partially disrupted. (B) Many nucleocapsids common to a viral envelope with envelope intact or partially disrupted.

in the so-called FP plaques (11, 12). The alternative plaque type is the MP type which is composed of many polyhedra per nucleus and many bundles of enveloped virus per polyhedron. Studies by Potter et al. (13) have shown that if virus picked from an MP plaque is passed serially in cell culture, the MP plaque type is the predominant type in early passages, but by passage level 25, FP is the predominant type. If the FP plaque is picked and passed likewise, it remains homogeneous. It was postulated that NPV undergoes mutation and selection to the advantage of the FP phenotype. This hypothesis may indeed be correct, but perhaps in addition, it would be wise to consider whether or not the phenotype of the infecting virion plays a role in the FP/MP phenomenon. Because of the multiple forms commonly produced in a single infected cell, the technique of picking a plaque could very well yield mixed phenotypic forms of the "purified" virus.

Consideration of the FP plaque phenomenon touches upon a very worrisome aspect of our method of assaying for infectivity by polyhedra production. Ramoska and Hink (14) reported that when they examined FP plaques using electron microscopy they found that the majority of infected cells contained no polyhedra whatsoever. We should consider the possibility of virus production without concomitant polyhedra production and what that implies for our *in vitro* handle on measuring infectivity. In 1971, Ignoffo, Shapiro, and Hink (15) reported that an established line of *Heleothis zea* ovarian cells could be infected by a *Heleothis zea* NPV and produce infectious virus but no polyhedra. Knudson and Tinsley (6) have suggested that *Spodoptera frugiperda* cells grown to confluency are incapable of producing cytopathic effect upon NPV infection. Further, James Vaughn observed that a special cell line of *Porthetria dispar* infected with *P. dispar* NPV showed no sign of CPE, yet when those cells were examined with an electron microscope, numerous intranuclear enveloped viral nucleocapsids were seen (personal communication). More recently, studies in Max Summer's lab have revealed that less than 0.5% of a culture of *Bombyx mori* cells infected with *Autographa californica* NPV contained polyhedra at 48 hours post-infection, yet when the culture medium was assayed for infectivity on TN-368 cells, about 10^6 PFU per ml were found. Further, when these cells were examined using an immunoperoxidase technique, less than 0.5% reacted with anti-polyhedrin, while over 90% reacted with anti-alkali-liberated *M*NPV.

Results such as these indicate that polyhedra production is not always a reliable indication of NPV infection and replication. The recognition of this leads to the realization that granulosis virus and *S*NPV may be infectious in cell culture after all, but thus far have gone undetected because of the lack of polyhedra production. Similarly, since polyhedra production differs with the line and subline of host cell, as well as with the infecting NPV, we may not be assessing our host ranges accurately. It is recognized that some investigators have used electron microscopy to assess GV and SNPV infectivity in some *in vitro* systems, but the complexity, cost, and time-consuming nature of this technique make it of limited use as a screening tool for many viruses in many different cell lines. In short, we urgently need to establish an assay that reveals the replication of the nonoccluded as well as the occluded virus. Without such an assay the standardization of biological activity in cell culture is equivocal and very limited. Perhaps the best indicator system we currently have is the Hink-Vail plaque assay (10) using TN-368 cells, and it was shown that sublines of these cells vary at least 75% in their capabilities of revealing infectious virus (16).

In summary, in order to improve the level of standardization of NPV biological activity, we need to take a harder look at the infectious capabilities of the different nonoccluded forms of virus. We need to be cognizant of the fact that we are working with a mixed form virus, and we need to establish an assay that is more closely tied in with virus replication than is polyhedra formation.

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DISCUSSION

SMITH: During the process of budding does the particle pick up any of the host nuclear or cytoplasmic membrane? Has anyone examined the structure and composition of the membrane?

VOLKMAN: Not yet. To answer your first question, we assume that the particle probably does.

SMITH: And this membrane is or is not present in the occluded virus?

VOLKMAN: There is an envelope, but it is not the same. The envelope of the occluded viruses is obtained intranuclearly.

RAPP: The major difference between the occluded and the nonoccluded virus is the fact that they occur singly or in pairs. Is there any other difference? Is there an antigenic difference? Is there some kind of biochemical difference that you can pinpoint for us? That alone would make them genetically mixed. Is there any evidence that suggests that they are genetically different?

VOLKMAN: There is no evidence that I know of that suggests they are genetically different, although I do not believe anyone has taken a critical look at that possibility. I think most people assume they are genetically the same, but they are phenotypically different.

HARRAP: I think we have one assay already, other than plaque assay. We can use virus particle antiserum on infected cell cultures. As I mentioned before, we can detect the infected cells at 24 hours; so, if you tested again in 48 hours, you could show the numbers increasing, etc. That is one alternative for looking at the presence of virus actually in the infected cell. I wondered if you had looked at possible different levels of infectivity with the different degrees of envelopment of the nucleocapsids. This is something we have been thinking about.

VOLKMAN: Yes, we have done only one study. We compared two nucleocapsids or more vs. single nucleocapsids from alkali-liberated preparations. We found there is very little difference in the level of infectivity between those two groups as assayed both by the plaque assay in cell culture, and _in vivo_ by _per os_ and intrahemocoelic administration.

(Tape change: some discussion missed.)

SUMMERS: For occluded enveloped nucleocapsids, the envelope is obtained in the nucleus and not by budding through the nuclear or plasma membrane. Is there a similar process that occurs with vertebrate viruses? The source of the virus envelope is obviously fundamental to differences in the physical, biological, and serological properties of the two forms of virus-occluded enveloped nucleocapsids and nonoccluded particles. This can be referred to your earlier question which you persistently bring to our attention -- what are we working with?

VOLKMAN: I would like to emphasize again that there seem to be at least three sources of envelopes: intranuclear _de novo_ synthesis, budding through the intranuclear membrane into the cytoplasm, and budding through plasma membrane.

RAPP: There are viral systems with large amounts of proteins and crystalline arrays but no envelope material. The adenoviruses are an example. The question is whether insect cells normally have that type of nuclear material, as it is not normally present in the mammalian nucleus. Has anyone examined that?

SUMMERS: Not definitively.

Pathogenic-Invertebrate Viruses: *In Vitro* Specificity

Dennis L. Knudson, Ph.D.
Yale University School of Medicine
New Haven, Connecticut

Invertebrate-associated animal viruses have been recently defined as being comprised of two distinct groups based on a criterion of pathologic effect (1). One group, arthropod-borne viruses (arboviruses), uses invertebrate vectors for biological transmission, replicates in invertebrates with little deleterious effect, and replicates in vertebrates with varying degrees of pathology. The second group, pathogenic-invertebrate viruses, is characterized by replication in invertebrates with pathologic effect and by the absence of any demonstrable pathology in vertebrates. Invertebrate-associated animal viruses such as Baculoviridae, cytoplasmic polyhedrosis viruses of Reoviridae, entomopoxviruses of Poxviridae, iridoviruses of Iridoviridae, and picorna-like viruses of Picornaviridae constitute the pathogenic-invertebrate virus group. This discussion of *in vitro* specificity will be restricted to this latter group of viruses, with particular emphasis on the Baculoviridae.

Both invertebrate and vertebrate continuous cell lines have been employed in assessments of *in vitro* specificity of pathogenic-invertebrate viruses, and the specificity of these viruses in vertebrate cell lines has been reviewed previously (2-4). The general consensus indicates that there is little evidence for the replication of pathogenic-invertebrate viruses in vertebrate cell lines with the possible exceptions of some of the icosahedral viruses. In contrast, pathogenic-invertebrate viruses do replicate in many of the invertebrate cell lines (1, 5). The existing data on the host range of pathogenic-invertebrate viruses in invertebrate cell lines are compiled in Tables 1 to 6. Both the positive demonstrations of virus replication and the negative results are recorded in the tables versus the appropriate invertebrate cell line.

It is tempting to speculate as to the significance of these data on the apparent *in vitro* specificity of pathogenic-invertebrate viruses. For example, the picorna-like virus seems to be specific for dipteran cell lines, and the entomopoxviruses and baculoviruses seem to be specific for lepidopteran cell lines. In contrast, the cytoplasmic polyhedrosis viruses and iridoviruses seem to lack *in vitro* specificity because they may replicate in both dipteran and lepidopteran cell lines. The data on Baculoviridae reveal that the *in vitro* replication of granulosis viruses has not been demonstrated. Furthermore, a few of the nuclear polyhedrosis viruses seem to exhibit an *in vitro* host range with a specificity which may reside at a familic or generic level. *Autographa californica* nuclear polyhedrosis virus (NPV) and *Trichoplusia ni* NPV appear to be two exceptions because they replicate in cell lines derived from lepidopterans of several different families.

Unfortunately, these speculations are premature and based on too little data. The "not tested" or zeroes in the tables outnumber the tested accounts, and furthermore, some of the tested accounts are recorded as personal communications or unpublished reports. This latter point becomes particularly apparent when examining the literature of the pathogenic-invertebrate virus specificity in vertebrate *in vitro* systems. Additionally, it is difficult to assess the significance of an unquantified negative result.

In assessments of *in vitro* specificity and in the interpretation of such data there are several parameters that must be clearly defined or, at least, considered. A methodology, for example, should be employed by which both the positive and negative results can be quantified, that is, relative to an internal standard such as a homologous system. The nature of the inoculum should be stated, and its biological activity should be quantified. The conditions of the test must be noted, particularly when there is deviation from the homologous system. Pertinent conditions to be considered and reported would include temperature, media, pH, and the growth phase of the test cells, such as log growth or stationary phase. The level of sensitivity in the detection of a viral effect on the cells must be clearly stated.

TABLE 1. PATHOGENIC-INVERTEBRATE VIRUSES: HOST RANGE IN INVERTEBRATE CELL LINES

	Cell line*							
	Diptera			Lepidoptera				
	A. aegypti	*A. albopictus*	*D. melanogaster*	*E. acrea*	*L. dispar*	*S. frugiperda*	*T. ni*	Ref.
Picornaviridae								
Picorna-like								
Cricket paralysis virus	0	+	+	0	0	-	0	†
Reoviridae								
Cytoplasmic polyhedrosis viruses (CPV)								
Aedes sollicitans CPV	0	+	0	0	0	0	0	(5)
Malacosoma disstria CPV	+	0	0	0	0	0	0	(6)
Trichoplusia ni CPV	0	0	0	+	+	+	+	(5, 7)

*+ = virus replication demonstrated; - = no virus replication demonstrated; and 0 = not tested.

†P.D. Scotti and J.F. Longworth (personal communication).

TABLE 2. PATHOGENIC-INVERTEBRATE VIRUSES: HOST RANGE IN INVERTEBRATE CELL LINES

	Cell line*						
	Diptera		Lepidoptera				
	A. aegypti	*A. stephensi*	*A. eucalypti*	*E. acrea*	*L. dispar*	*T. ni*	Ref.
Iridoviridae							
Iridovirus							
Chilo iridescent (type 6)	+	+	+	0	+	+	(5, 8-10)
Mosquito iridescent (type 3)	+	0	0	0	0	0	(11)
Sericesthis iridescent (type 2)	+	0	+	0	0	0	(8, 9, 12)
Tipula iridescent (type 1)	+	0	+	0	0	0	(12, 13)
Poxviridae							
Entomopoxvirus							
Amsacta moorei	0	0	0	+	+	0	(12)

[*] + = virus replication demonstrated; - = no virus replication demonstrated; and 0 = not tested.

TABLE 3. PATHOGENIC-INVERTEBRATE VIRUSES: HOST RANGE IN INVERTEBRATE CELL LINES[†]

	Cell line[*] Lepidoptera		
	H. zea	*S. frugiperda*	*T. ni*
Baculovirus			
Granulosis virus (GV)			
Heliothis zea GV	-	-	-
Spodoptera frugiperda GV	-	-	-
Trichoplusia ni GV	-	-	-

[*] - = no virus replication demonstrated.

[†] R.H. Goodwin (personal communication).

TABLE 4. PATHOGENIC-INVERTEBRATE VIRUSES: HOST RANGE IN INVERTEBRATE CELL LINES

	Cell line[*] Lepidoptera			
	H. zea	*S. frugiperda*	*T. ni*	Ref.
Baculovirus				
Nuclear polyhedrosis virus (NPV)				
single embedded type				
Heliothis zea NPV	+	-	-	(14, 15)[†]
Trichoplusia ni NPV	-	-	+	(14, 15)[†]

[*] + = virus replication demonstrated; - = no virus replication demonstrated; and 0 = not tested.

[†] R.H. Goodwin (personal communication).

TABLE 5. PATHOGENIC-INVERTEBRATE VIRUSES: HOST RANGE IN INVERTEBRATE CELL LINES

	Cell line*			
	Diptera			
	A. albopictus	*A. stephensi*	*A. tritaeniorhynchus*	Ref.
Baculovirus				
Nuclear polyhedrosis virus (NPV)				
multiple embedded type				
Autographa californica NPV	–	–	–	†
Spodoptera frugiperda NPV	–	0	0	†
Trichoplusia ni NPV	–	–	–	†

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[*] + = virus replication demonstrated; – = no virus replication demonstrated; and 0 = not tested.

[†] R.H. Goodwin (personal communication).

TABLE 6. PATHOGENIC-INVERTEBRATE VIRUSES: HOST RANGE IN INVERTEBRATE CELL LINES

	Cell line* Lepidoptera											
	A. eucalypti	B. mori	E. acrea	H. zea	L. pomonella	L. dispar	M. disstria	M. sexta	S. frugiperda	S. littoralis	T. ni	Ref.
Baculovirus												
Nuclear polyhedrosis virus (NPV) multiple embedded type												
Antheraea pernyi NPV	0	0	0	0	0	(+)	0	0	0	0	0	†
Autographa californica NPV	0	0	+	(+)	-	(+)	0	(-)	+	0	+	†† (5,16)
Bombyx mori NPV	+	+	0	0	0	(+)	0	0	0	0	0	†‡ (17)
Choristoneura fumiferana NPV	0	0	0	0	0	0	+	0	0	0	0	(21)
Galleria mellonella NPV	0	0	0	0	0	(+)	0	0	0	0	+	† (6)
Heliothis armigera NPV	0	0	0	+	0	0	0	0	-	0	-	†
Lambdina fiscellaria somniaria NPV	0	0	0	0	0	0	+	0	0	0	0	(18)
Lymantria dispar NPV	0	0	0	-	0	+	0	-	-	0	-	†
Orgyia pseudotsugata NPV	0	0	0	-	0	+	0	-	-	0	-	†
Spodoptera exempta NPV	0	0	0	0	0	0	0	0	+	0	0	‡
Spodoptera exigua NPV	0	0	0	0	0	0	0	0	+	0	0	‡
Spodoptera frugiperda NPV	0	0	0	-	0	0	0	0	+	+	-	† (19,20)
Spodoptera littoralis NPV	0	0	0	0	0	0	0	0	+	+	0	(19)
Trichoplusia ni NPV	0	0	0	(+)	0	(+)	0	+	+	0	+	† (21)

* + = virus replication demonstrated; - = no virus replication demonstrated; 0 = not tested; and ( ) = conflicting or unconfirmed reports.

† R.H. Goodwin (personal communication).

‡ D.L. Knudson (unpublished data).

I would like to present an example of a methodology by which both the positive and negative results can be quantified in assessments of *in vitro* specificity of baculoviruses. The *in vitro* host range of six NPVs isolated from insects of the family Noctuidae was examined in five lepidopteran continuous cell lines. Three of the cell lines were derived from tissues of insects from the family Noctuidae. While the remaining two, the *Lymantria dispar* cell line (IPLB-PD-65Z) and the *Choristoneura fumiferana* cell line (IPRI-CF124), were derived from tissues of insects from the families Lymantriidae and Tortricidae, respectively. The cell lines were all grown in the same medium, TC100 (22), and the viral inocula were all low-speed clarification supernatants of infected cell cultures (23).

The test simply consists of an endpoint dilution titration of a given NPV in the various cell lines. A dilution series of the virus was made, and 0.1 ml of each dilution was introduced in the test system containing a specific number of cells which came from log growth phase cell cultures. The same dilution series was also titrated into the homologous cell system. The homologous system was operationally defined as the cell line in which the viral inoculum was grown. After an incubation period of five days, the titration was scored for the characteristic baculovirus cytopathic effect; that is, the formation of polyhedra in the nuclei of the cells (22). The 50% tissue culture infective dose ($TCID_{50}$) per milliliter of inoculum was calculated from the homologous system and the test cell line or heterologous system. The efficiency of the virus titration or assay in different cell lines was expressed relative to the homologous system. Table 7 represents the data on the *in vitro* host range of the six baculoviruses in the five lepidopteran cell lines presented as the efficiency of assay, the heterologous $TCID_{50}$ per ml exponent over the homologous $TCID_{50}$ per ml exponent. The expression equal to or less than ($\leq$) 1.5 in the table is indicative of the lower level of quantification of these tests. A tenth of a ml of a 10^{-1} dilution gave no cytopathic effect in the test system, and therefore, the $TCID_{50}$ per ml would be $10^{1.5}$. These data allow comparisons to be made of the virus titer between a given heterologous cell line and homologous cell line. A simple normalization step, however, allows comparisons to be made between the cell lines and the different viruses. The data from Table 7 which have been normalized and expressed as $\log_{10}$ function are shown in Table 8. The values noted in Table 8 represent an index of the efficiency of the virus assay in the heterologous cell line relative to the homologous cell line, or simply, an efficiency index. The efficiency indices are useful for several reasons. They allow direct comparisons to be made because the indices represent constants which should only vary by

TABLE 7. *IN VITRO* HOST RANGE OF BACULOVIRUSES: EFFICIENCY OF ASSAY*

Virus†	Lepidopteran cell line				
	C. fumiferana (IPRI-CF124)	*L. dispar* (IPLB-PD-65Z)	*S. frugiperda* (IPLB-SF-21)	*S. littoralis* (UIV-SLC)	*T. ni* (TN368AMC1)
Autographa californica NPV-S/TN5	$\frac{\leqslant 1.5}{7.0}$	$\frac{6.5}{7.0}$	$\frac{6.7}{6.7}$	$\frac{5.0}{7.0}$	——‡
Spodoptera exempta NPV-S/SF2	$\frac{3.3}{7.8}$	$\frac{8.0}{7.8}$	——	$\frac{6.1}{7.8}$	$\frac{7.9}{7.6}$
Spodoptera exigua NPV-S/SF2	$\frac{2.7}{6.9}$	$\frac{7.0}{6.9}$	——	ND§	$\frac{7.1}{6.4}$
Spodoptera frugiperda NPV-S/SF2	$\frac{\leqslant 1.5}{5.1}$	$\frac{\leqslant 1.5}{5.1}$	——	ND	$\frac{4.4}{5.9}$
Spodoptera littoralis NPV-S/SLC2	$\frac{\leqslant 1.5}{3.8}$	$\frac{\leqslant 1.5}{3.8}$	$\frac{2.2}{3.8}$	——	$\frac{\leqslant 1.5}{3.9}$
Trichoplusia ni NPV-S/TN53	$\frac{\leqslant 1.5}{7.5}$	$\frac{7.3}{7.5}$	$\frac{7.2}{7.6}$	$\frac{2.9}{7.3}$	——

*Efficiency of assay = $\frac{\text{heterologous } TCID_{50}\text{/ml exponent}}{\text{homologous } TCID_{50}\text{/ml exponent}}$

†Virus = genus species, nuclear polyhedrosis virus, infected cell culture supernatant, passaged in designated cell line n times.

‡—— = homologous system.

§ND = not done.

TABLE 8. *IN VITRO* HOST RANGE OF BACULOVIRUSES: EFFICIENCY INDEX*

Virus†	Lepidopteran cell line				
	C. fumiferana (IPRI-CF124)	*L. dispar* (IPLB-PD-65Z)	*S. frugiperda* (IPLB-SF-21)	*S. littoralis* (UIV-SLC)	*T. ni* (TN368AMC1)
Autographa californica NPV-S/TN5	≤-5.5	-0.5	0	-2.0	$\underline{0}$‡
Spodoptera exempta NPV-S/SF2	-4.5	0.2	$\underline{0}$	-1.7	0.3
Spodoptera exigua NPV-S/SF2	-4.2	0.1	$\underline{0}$	ND§	0.7
Spodoptera frugiperda NPV-S/SF2	≤-3.6	≤-3.6	$\underline{0}$	ND	-1.5
Spodoptera littoralis NPV-S/SLC2	≤-2.3	≤-2.3	-1.6	$\underline{0}$	≤-2.4
Trichoplusia ni NPV-S/TN53	≤-6.0	-0.2	-0.4	-4.4	$\underline{0}$

*Efficiency index = $\log_{10} \dfrac{\text{heterologous TCID}_{50}/\text{ml}}{\text{homologous TCID}_{50}/\text{ml}}$

†Virus = genus species, nuclear polyhedrosis virus, infected cell culture supernatant, passaged in designated cell culture n times.

‡$\underline{0}$ = homologous system.

§ND = not done.

the standard error of the titration. When the columns are examined, a comparison of the indices is indicative of the susceptibility of the cell line to the various NPVs that were tested, and the level of the susceptibility is quantified. When the rows are examined, a comparison of the indices reflects the replication potential of a virus in the cell lines that were tested, and likewise, this function is also quantified. For example, the low level of replication of _S_. _exempta_ NPV-S/SF2 in the _C_. _fumiferana_ cell line may not have been detected if the virus inoculum had a lower initial titer.

The data in Table 8 do indicate the degree of _in vitro_ specificity of baculoviruses in invertebrate cell lines using the noted levels of input test virus and using the detection system of cytopathic effect. Clearly, this approach only represents the first step in the assessment of the _in vitro_ specificity of baculoviruses. Undoubtedly, tests of increased sensitivity in the detection of viral replicative events in cells will represent the second and further steps in such specificity assessments.

These data are preliminary observations which will be reported elsewhere in greater detail.

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DISCUSSION

SUMMERS: You are comparing Autographa NPV/TN passage 5 with Spodoptera NPV-S/SF passage 2. In our hands, Autographa NPV passage 5 drops off significantly in its ability to produce polyhedra relative to passage 1 or 2, by a rather large percentage.

KNUDSON: This is in T. ni cells?

SUMMERS: Yes. I use that as a standard system. We expect comparable phenomena to occur in other cells, although maybe that is a wrong assumption. As you compare TN-5 with SF-2, would you expect there to be differing levels in the ability of that passage of the virus to induce polyhedra?

KNUDSON: I am not talking about the number of polyhedra in the cells, but clearly, that may be possible. I am just listing a group of viruses. For example, the T. ni passage 53 which I received from Dr. Faulkner should contain strictly FP virus. In our hands, we find there are many polyhedra using the Spodoptera cell line and they produce polyhedra very well.

SUMMERS: Still, even with plaques, it would be significantly different from early passage T. ni virus.

KNUDSON: I think that is the nature of this meeting. Are they going to be significantly different, and how may that best be determined? This is a system, for example, by which you could examine passage effects.

FIELDS: Have you been able to determine whether this is a surface restriction or if this is intracellular, and do you have any idea of dominance, say, of fusion? I ask this because it would be a powerful genetic tool in looking at host range differences among this important group.

KNUDSON: I have not had an opportunity to do the types of things that you asked about, but we got involved in this type of experimentation precisely for the reason that you mention, that is, the idea of using this for looking for host range mutants. We are interested in this particular area, and we wish to set up a system by which we could quantify the differences in host range and use that as a marker for the genetics.

STOLLAR: Is there any indication that serial high multiplicity passage leads to something like the generation of defective particles?

KNUDSON: There are a number of accounts that seem to clearly indicate that serial passage of the virus over a number of periods will produce something that is referred to as an FP type polyhedra. The degenerative change that takes place over multiple passage is the formation of polyhedra that are apparently devoid of virus. For example, this Trichoplusia ni NPV/TN 53 is an FP phenotype in the T. ni cell line according to Dr. Faulkner. In the titration, the classic sort of interference with high multiplicity input does not appear, but virus replication at high multiplicity does occur. So at the moment, there is no real evidence of a defective particle that is interfering. They are defective particles, perhaps, but they do not interfere.

PART IV
PANEL DISCUSSION

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Viral Pesticides: Implementation and Safety

Moderator: Victor Stollar, M.D.

STOLLAR: Five or six people will begin our discussion, which will be less formal than the presentations we have had up to now. Incidentally, we would like initially to limit the discussion to participants; later on other people at the meeting may join in.

I would like to make a few remarks before we hear from our first discussant. The question we are trying to focus on in this meeting is, "What is the potential harm of using insect viruses as insecticides?" What damage could they do to man and domestic or wild animals? Thus we must ask, "What is the capacity for replication of insect virus in vertebrate organisms? Can there be untoward effects of insect viruses even in the absence of complete replicative cycles? Could there be abortive infections? Could there be persistent low-grade infections?"

Perhaps we might be able to get some useful comparative information from observations made of various arboviruses in mosquito cell lines -- an area with which I am familiar.

I think we can rationalize this as follows. The arboviruses and, more specifically, various of the togaviruses and bunyaviruses are viruses that specialize in alternate replication and transmission between widely different organisms. This alternate transmission is required for their maintenance

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in nature. By evolution, they have adapted so that they can replicate well in very different kinds of hosts or species. Togavirus models could, I think, be useful because there has been a good deal of information acquired both at the level of the whole organism (mammalian or arthropod) as well as in tissue culture. In the case of a virus such as Sindbis, a great deal is also known about its molecular biology.

Let me very briefly run through some generalizations which can be made about viruses such as Sindbis, a representative member of the alpha virus subgroup of the togaviruses. As Dr. Knudson just stated, these viruses generally have potential for causing disease in vertebrate species and cytopathic effects in vertebrate cells; in contrast, they are generally noncytopathic in the insect host, whether that is the whole mosquito or a tissue culture. As Knudson pointed out, this is the inverse of what we see with the insect viruses. Perhaps if we knew why there is this interesting difference, why togaviruses can kill vertebrate cells, but not insect cells, it might help us understand not only how insect viruses kill insect cells, but perhaps also what their potential might be for harmful effects in higher organisms.

Another generalization is that arboviruses, because of selective pressures exerted during the course of evolution, have developed the capacity, more so probably than most other viruses, to replicate over a very wide temperature range -- from 20° to 40°. However, we must point out that in each system the optimum temperature for virus replication is dictated by the optimum temperature for the host cell. I think when we are considering the potential of insect viruses, the effects of temperatures on virus replication in the various cell lines will have to be considered.

Arboviruses are enveloped viruses. They bud off from various cellular membranes and in the process incorporate carbohydrates and lipids derived from the host. Thus, a togavirus that is produced in a mosquito cell would have a very different composition as far as its carbohydrates and lipids from a virus produced in a mammalian cell. For instance, one major difference that we have shown is that in contrast to virus grown in mammalian or chick cells, Sindbis virus grown in mosquito cells contains no sialic acid. But the variation in carbohydrate and lipid composition, in the case of Sindbis virus at least, has little, if any, effect on the infectivity of that virus. In other words, viruses are equally infectious and have the same particle to PFU ratio, whether they are grown in mosquito cells or mammalian cells. Similarly, we have shown that the host cell in which the virus is grown had no detectable effect on the antigenic properties of the virus. I think this might bear on some of the questions that have arisen today.

In other words, when we grew virus in chick cells and in mosquito cells, purified the viruses, produced antisera against each, and reacted each antiserum with the virus grown in each cell type -- we couldn't differentiate between the virus grown in the chick cells and that grown in mosquito cells.

We said there is no CPE produced by togaviruses in insect cells. That is a rather sweeping generalization, and there are a number of exceptions. There is at least one report of some cytopathic effect in the mosquito produced by Semliki Forest virus. If we turn to the flaviviruses (dengue Japanese encephalitis, yellow fever viruses), these viruses can produce quite a massive cytopathic effect in mosquito cell cultures. Furthermore, we have recently obtained cloned populations of mosquito cells, which in contrast to all uncloned populations, and to other clones, do show marked cytopathic effect after infection with Sindbis virus.

Therefore, it appears that some of the earlier generalizations made and accepted by people working with arboviruses will not hold up; this leads me to believe that as we study more about the replication of insect viruses both in insect cells and in cells of higher organisms we may be in for many surprises.

I also would like to emphasize, from our work dealing with insect cell cultures, that we have been strongly impressed by the importance of careful characterization of the cell types and cloning of the cell types.

We have seen in the literature, reports of different clones or strains of mosquito cells that give equivalent yields of viruses but which differ very markedly in the response of the cell to the virus. This means that in looking at insect viruses in mammalian cells, many different cell lines will have to be tested. That might be a topic for further discussion. I think it is important that the insect cell lines that people work with be well characterized, and if possible, cloned. I think it would be useful to have them karyotyped because there has been at least one instance where people thought they were working with a mosquito cell line but were in fact using a moth cell line.

The media used for insect cell lines are usually undefined and rather complex. In the case of several mosquito cell lines, it has been possible to adapt them to a simpler medium, namely Eagle's medium supplemented with fetal calf sera. This adaptation facilitates many kinds of experiments involving radioisotopes and might be useful to try in the case of the various Lepidopteran cell lines.

Lastly, we have to be aware that latent or unsuspected viruses may be present in our insect cell cultures. There are examples of this already reported -- perhaps some examples will be described by the various participants or, if people are interested, we can describe some of the examples of unsuspected or latent contamination in some of the mosquito cell lines.

We'll now turn the discussion over to Dr. Shope.

SHOPE: Several of the participants have urged that someone introduce or reintroduce the concept that one of the needs in the invertebrate pathogenic virus field is the cataloguing of some of the information that has been presented this morning, as well as information from investigators throughout the world. I have had some experience since 1959 with a similar cataloguing exercise with the arboviruses.

I have with me the International Catalogue of Arboviruses. One might conceive of an international catalogue of viruses pathogenic to invertebrates formulated in a similar manner. The Catalogue of Arboviruses evolved from a group of people sitting down in 1959, very similar to this group, with a field of viruses, which in its technological development was not as far as we are today with baculoviruses and the other invertebrate pathogenic viruses. The decision was made to draw up some format to gather information and then to collaborate, share information, and have a sort of information exchange, first on an informal basis. Later on, this evolved into a published book that is now in its second edition. This contains something over 360 catalogued arbovirus serotypes.

The form that is filled out by the person who catalogues individual viruses contains information such as: the name and reference type of the virus, the origin and method of isolation, virus properties, properties of its DNA or RNA, and reports of virion and nonvirion peptides. The form also lists the morphology; hemagglutination; antigens and antigenic relationships; biological characteristics, such as animal host-range; tissue culture susceptibility; experimental models; the geographic distribution; and a list of references. Also, in the case of arboviruses, one of the categories reported is the disease in vertebrates that might or might not be applicable to the viruses pathogenic to invertebrates. I will pass around the catalogue and the latest version of the filled-in catalogue page for people to look at. Mr. Longworth sat down to see if he could fill in this information with one or more of the viruses with which he was familiar.

LONGWORTH: With Dr. Shope's help, we looked at the form and condensed it to get a working information sheet, which investigators throughout the world can fill in. So if an investigator wishes to compare his isolate with another, this is one way of getting information. How such a catalogue should be organized can be left to discussion.

STOLLAR: When a virus is isolated and is submitted, does it have to be confirmed independently or is only one person involved?

SHOPE: In a working sense, what we initially were interested in was if someone had an isolate that he felt was different from anything already in the catalogue, then he was free to catalogue it, not in a published form, but in an informal form. We simply encouraged people to submit information. When it became obvious in certain cases that people were submitting information on the same agent, they would get together to combine their information.

HARRAP: How was it funded?

SHOPE: Initially, in 1959, there was a small grant from the Rockefeller Foundation, somewhat under 20 thousand dollars, which paid for the paper and postage and some secretarial help. The actual professional work was all volunteer. The catalogue activity was taken over by the National Institutes of Health, and subsequently, by the Center for Disease Control. It is now a full-time activity of the Center for Disease Control, which is part of HEW (U.S. Department of Health, Education, and Welfare).

HEIMPEL: It is all very well to record the differences in viruses and the various germ plasms that are isolated, but there should be a concerted effort by everybody to establish some sort of system to preserve these. Now, the USDA (U.S. Department of Agriculture) is looking into this, rather slowly but thoroughly, and one of the proposals is to try to commit funds permanently to get the American Type Culture Collection, which is a private organization, to store insect cells and insect pathogens and plant pathogens. I've made my plea all over the world, the last time in Rome to the FAO (Food and Agriculture Organization), which now has a scheme for preserving plant germ plasm, and got a rather positive response from them, that is, they said they would look into it. But if all those present would support a drive to preserve these organisms in perpetuity, I think it would be of great use to us all. Actually, it worries me to see people working with what is called *Autographa californica* virus, and they really don't know if that is what they are working with because the noctuid viruses are so close. If we had a standard source, we could all start from the same line.

STOLLAR: It seems that the time has arrived for some serious effort at cataloguing; I'm not quite sure how this will be done, or who will take the responsibility for it.

SHOPE: I agree, and in the case of arboviruses this can be used as an analogy. The American Type Culture Collection now contains 116 arboviruses, and the decision as to which arboviruses would be selected in reference type was made on the basis of the recommendation of the group that was cataloguing the viruses, so that the two functions would complement each other.

STOLLAR: Something along this line has been started with various insect cell lines. Dr. Fred Hink has put together a catalogue of over 100 insect cell lines, and he's made this catalogue available to everyone.

McCARTHY: Considering the classification of the viruses, I don't think enough fundamental knowledge is known about the viruses to classify them as such, as yet, in terms of the nucleic acids and serological responses.

MILLER: There is an excellent method for identification of baculovirus, and that is the restriction endonuclease analysis of the viral DNA. It is quite possible to do this, and a very good catalogue could be made of restriction enzyme patterns of the various viruses. This will be one way of distinguishing viruses other than the host-range morphology and other types of identification that are currently available.

McCARTHY: I wasn't saying that there is no method available, just that the data haven't been collected yet.

SUMMERS: I would agree with that, but we have various levels of identification with which to deal. One of the routine procedures is a serological approach and another is restriction endonuclease or physical mapping of viral genomes; at the moment we are dealing with our serological problems. Later we will deal more specifically with DNAs and with problems and approaches in that area. I would like to reinforce what Bill McCarthy said, not so much in terms of DNAs, but in terms of what we know about baculoviruses, their complex structure, and so forth. Is the state of our art good enough today to actually initiate a cataloguing system?

SHOPE: I think this is the beauty of an informal information exchange as the initial step, in that you don't have to feel that you must have the data needed to establish it as a type virus. All you do is put the available data down on paper and let other people decide whether to confirm it.

IGNOFFO: There is a cataloguing system available from Dr. Martignoni.

SUMMERS: We need to look at that catalogue in terms of how it can be expanded into the scheme given to us by Dr. Shope. If you look at Martignoni's catalogue carefully, it cites insects in terms of genus and species, and virus diseases that have been recorded as associated with the insect.

SHOPE: I am aware of Dr. Martignoni's catalogue. What I had in mind wasn't meant to supplant what he's done, rather, to continue it and supplement it with a great deal more information, for instance, on some of the physical properties.

IGNOFFO: Nor did I mean to exclude that as more and more information is available, you try to characterize it as much as possible and put it into a form that everyone can use. All I meant to say is that there is an attempt to catalogue right now on the basis of what information is known -- insect viruses as they are found. It's true in some instances it's very fragmentary and deals with initial isolation -- but that's how knowledge is accumulated.

HARRAP: The cataloguing that Martignoni has published is formulated on the basis of virus host. This is the difference we should emphasize, developing a catalogue formulated on the basis of the virus. The problem is what properties of the virus do you use? In the last four years, there have been national attempts to establish an insect virus classification working group. It has just been very difficult to get it off the ground. Perhaps the idea of simply filling out a form for a given virus is the best way of starting.

WOOD: In our rush to characterize and identify these viruses, we have ignored certain considerations. One is the sources of these viruses. In some cases, a single virus has been isolated independently by several laboratories, or from a handful of insects of the same species. How many different viruses are really even in a single insect? How many variants of the same virus are present? I think we have a real problem here, particularly when we try to relate our information to potential health hazards.

Cleanup procedures of the virus populations really only occur through a filtering process as we pass these viruses through insects and tissue cultures. I think a lot of attention should be paid to cloning these viruses. We now have sufficient facilities and techniques for cloning of viruses which we are particularly interested in. We should work with clones particularly in investigating the physical nature of the particles and immunology. We also have the opportunity of looking at the biological properties of different clones; possibly we will find clones which have a greater efficacy in terms of biological control.

We've been looking at these viruses chemically and physically, but not really biologically. We are worried about health hazards primarily, and not about characterization. We don't really understand what goes on in an insect cell, as far as what the viruses are doing. So how are we going to know what's going on in a nontarget cell? What are the possibilities of a potential hazard with these viruses? I think this is a very important point, and I think we have to get into insect cells and look at the viruses to see if there is a potential for abortive infection or an introduction of some of the viral genome into the host cells.

McCARTHY: Since these are known as insect viruses, what happens when you put cloned virus back in the lung? How do you know what you put in is what you get out?

WOOD: Once you have a clone that has a marker with a particular property, then if you pick up a latent virus, either in the insect as you have to pass it through, or in tissue culture, you have a tag to know that something's gone wrong.

RAPP: There is a tendency for investigators to take a virus from its natural system and to start passing it in the animal or in vitro. What everyone looks for, of course, is a virulent virus because they are looking for cytopathic effects, they are trying to get something that grows rapidly to high titer. This is often far removed from the initial organism, but is the one that is likely to be used as a pesticide. Some attention ought to be given to looking for markers of attenuation of some kind. If there is an outbreak in the field, then one can relate back to whether the outbreak occurred naturally, or whether it is the field virus, that is, a sprayed virus that produced it. This is something that turned out to be very valuable, for example, in the polio vaccination campaign in this country.

Furthermore, it seems to me that there should be an attempt made to keep isolates of viruses close to fresh isolations and prototype viruses frozen down, so that they can be compared, whether it's polypeptides or a restriction endonuclease analysis of DNA, with what has developed subsequently down the line. You find, for example, if you look for human or animal isolates in this country, fresh isolates are very hard to find. And if you do find them, most are less virulent, considerably less cytopathic in cell cultures, than those viruses that have been developed to work with. Yet those may in fact be more dangerous. So the early isolates have to be maintained, and this is a good time to start freezing down fresh isolates that have not been passed and to get multiple isolates to compare.

COLLINS: Since we are dealing with the question of how these viruses may change with passage, one point that has not been mentioned at all today is, has anyone looked at the status of endogenous virus information in these normal insect cell lines that are being used to propagate the virus? Is there any indication of antigen expression related to these viruses of nucleic acid?

GRANADOS: I would like to comment on possible adventitious agents, mycoplasma, or viruses in cell lines currently used by insect virologists. Recently we came across a problem associated with one of the first insect cell lines that Hink established, the _Heliothis zea_ IMC-HZ1 cell line that is widely distributed in the U.S., and probably abroad, too. We had difficulty growing it, as we did with a similar cell line obtained from Knudson. We looked at these with an electron microscope and found low levels of infection with a nonoccluded baculovirus type particle. We infected TN-368 cells with the virus. We found a very dramatic infection rate to the extent that within three days postinoculation, all cells were lysed. I am sure that if people were to take a good look at what is happening to these cultures before they get into detailed virus work, more of these adventitious viruses might be picked up.

KNUDSON: I am very glad that Granados has had a chance to look at the _H. zea_ cell line. This is a cell line that I first received many years ago while I was at Oxford, and it is for the very reason that we had difficulty in handling that cell line that we did not use it for much of our virological work.

We carry a number of the Lepidoptera cell lines and, in looking for endogenous agents, we have not by any means examined them all. Certainly in the _S. frugiperda_ cell line that I work with, I have never encountered anything by electron microscope. However, we know that is only one level of sensitivity. On the other hand, Longworth has detected adventitious agents in the _S. frugiperda_ cell line that I sent him.

STOLLAR: With the mosquito cell lines, there certainly has been clear evidence of unsuspected contamination, for example, in the report by Buckley and co-workers. This was first demonstrated in EM sections which showed that their strain of the Aedes albopictus cell line was full of crystalline aggregates, subsequently shown serologically to be chikungunya virus.

In Australia, Russell Regnery found an Aedes albopictus cell line contaminated with what proved to be Semliki Forest virus. In another instance, we worked in our laboratory with an Aedes aegypti cell line that looked perfectly fine. It grew well and there was no suggestion or evidence of infection, but medium from that culture, when put on the Aedes albopictus cell line, caused massive cell fusion within two days. Subsequently, we purified and characterized to some extent an agent that appears to be a togavirus, but so far it has not been further identified. It does not seem to be an alpha or flavivirus.

These studies were done with the help of Dr. Casal at Yale. So here we have an agent not yet identified of which we would not have become aware because it does nothing to mammalian or chick cell cultures, even at lower temperatures. It seems, therefore, that the only indicator cell line we have for this agent from the Aedes aegypti cell line is the Aedes albopictus cell line.

HARRAP: We have detected vast arrays of small spherical particles in T. ni cells from Howard Stockdale at Shell. Because of this, little work has been done with the T. ni system.

SUMMERS: What was the size of that particle?

HARRAP: It was the size of a parvovirus.

SMITH: Experience with vertebrate viruses has shown that superinfection brings up a line with a particular virus, which may activate an occult, latent, or even integrated and completely silent virus. Has this been observed, and is there any way to test for it?

HARRAP: I can't think of an instance in cell culture, but we have an intriging instance in insects which it might be useful to describe. The Spodoptera littoralis insect cultures have been growing on a synthetic diet in our laboratory for some years now. We did not do any cross-transmission tests with the other Spodoptera viruses at first because we did not have any criteria for checking what we might get out. As soon as we had some biophysical data on the viruses allowing identification, we did this work, and sure enough, the viruses seemed to be cross-transmissible. But then when we started to look at the products of the cross-transmission tests, surprising results were found. When we fed *Spodoptera frugiperda* NPV to our *Spodoptera littoralis* larvae, and we looked at the polyhedra resulting from this cross-infection, they turned out to be *Spodoptera littoralis* polyhedra by several criteria; for example, GC content, structural protein profile, and the antigen-antibody precipitation pattern in immunodiffusion tests. Even the profile on the sucrose gradient after alkali release of virus from polyhedra was characteristic of *S. littoralis* virus in larvae killed as a result of feeding of *S. frugiperda* virus. Initially, we thought the *S. frugiperda* inoculum must be a mixture of the two viruses, and that perhaps we had some sort of selection system occurring.

We got an alternative stock of *S. littoralis* larvae from Israel, did the same tests and found that we could not get infection in these larvae. However, larvae from the two insect cultures died with the same sort of kinetics with the homologous virus. So then we came to the conclusion that in some way we had activated virus latent in our original *S. littoralis* insect culture. We attempted a variety of temperature shifts and other stress phenomena on the insects but we could not induce them to die of virus. We have never seen frank expression of disease in the insects themselves, and we can't detect the NPV in them in any way, but it seems to be there.

We have now achieved the same result by feeding another NPV, that of the *Heliothis armigera*, to this insect stock. The difference here between the two viruses is even more marked. The *Heliothis armigera* virus is a singly enveloped rod, and the *Spodoptera littoralis* is an enveloped bundle of nucleocapsids; so all one has to do is to run a gradient to find out which virus is present. It seems as though we may have a model situation here for looking at a latent or subclinical NPV infection in an insect population.

McCARTHY: My laboratory is uniquely connected to a group that is carrying on a lot of field work. They spray 35-acre plots with gypsy moth NPV, and they are also treating 2- and 4-acre cabbage plots with *Autographa californica* and *Pieris rapae* granulosis. I think the same considerations are applied for characterizing baculoviruses -- for instance, serology and DNA hybridization. Some of these same considerations can be applied to field formulations. I am an interested spectator, that's all, on these field problems. But when I see what goes on with the formulations that are made up, I think it is a good consideration for safety and toxicity regulations.

The preparations that we use for characterization, and most everybody does, bear little relation to the preparations that are being used in field work. Many of them contain a certain percentage of insect parts -- in the case of gypsy moth, they are highly allergenic to some individuals, and they have certain levels of bacteria. Then comes the addition of stickers, spreaders and UV protectants, and all these are sprayed out into the environment. I think that quality control regulations have to be instituted along these lines, and this eventually leads to the quality control of the virus that you are using in the first place, which in turn leads to the specific definition of the type of virus that you are using -- biologically, chemically, and genetically. So not only does the strict definition of your baculovirus become important, but that is carried all the way up the line to the use of that virus in formulation, and it must be strictly defined also.

STOLLAR: Could you fill us in a little on commercial formulation?

McCARTHY: I am not really that familiar with commercial formulation.

IGNOFFO: Concerning the nonoccluded virus particles from the HZ-1 cell, I was wondering if any of you have checked for infectivity in noctuids hosts?

GRANADOS: Sometime today, my research assistants have fed a number of Lepidopterous species with this nonoccluded virus. Heliothis zea will be one of them.

IGNOFFO: Then you are familiar with the particular lines in question that have been infected with the DNA of the Heliothis zea NPV?

Viruses have been formulated in many different ways. The earliest and the easiest method was to grind up the virus-infected insect using water as the carrier. More recently, dry preparations of technical lots of virus have been prepared, using the following procedures: freeze-drying, vacuum-drying, acetone precipitation, air-drying, acetone precipitation with various adjuvants such as lactose, and spray-drying. Spray-drying (with or without adjuvants) has been successfully used to prepare viruses. Specific adjuvants are included to increase shelf life, sunlight persistence, sprayability, wettability, and to increase the physical properties of the virus, thus making it easier to apply in the field. Preparations of formulated virus are generally applied, using water as the carrier. Dusts also have been successfully used in the past. Current commercial preparations of virus are wettable powders that are applied using water as the carrier.

STOLLAR: There is no extensive purification of a specific virus?

IGNOFFO: Not to the level of purification required for biochemical or physical characterization. In the final analysis, if you can ensure that the virus in an unpurified technical formulation is safe (not toxic or pathogenic), then this is probably a better measure of potential risk than if you have tested a purified preparation which in the process of purification may have eliminated an entity which was unsafe.

STOLLAR: Would the cost of using more extensively purified virus be prohibitive?

IGNOFFO: I am not in a position to answer that. I can give you an opinion that it would be more expensive, but there are other people from industry present here who could give you an estimate of the cost of purification.

HOLOWCZAK: If the viruses are so specific and apparently relatively easy to come by, in theory at any rate, why haven't they, in fact, just taken over the field and replaced chemical pesticides completely at this point, when the chemical people have so many problems facing them, so to speak, in terms of carcinogenicity, etc.

IGNOFFO: A big disadvantage to viruses vis-a-vis chemical insecticide, which is probably true of most entomopathogens, is that they do not kill as quickly as chemical insecticides. A chemical insecticide, because it is a highly toxic, broad-spectrum agent, could eliminate pest caterpillars within hours of application. Biological insecticides cannot do this! Currently, most persons recommend that biological insecticide be used as the primary control method and that chemical insecticides only be used as a last resort when the farmer is facing an imminent loss situation. For example, if the farmer is in a situation where he would lose his crop in a matter of a few hours to days (the worms are so large that no disease would knock it down quickly enough), then a chemical insecticide should be used. We are recommending, however, that if chemical insecticides must be used, that they be prudently used, used at lower doses and only when no other recourse is available.

HOLOWCZAK: I assume in almost all cases they would have to be applied each year -- the viral pesticide each year.

IGNOFFO: Your assumption is partially correct, depending upon the ecosystem, and the level of controls which is required. In a row-crop system where plants grow at a rapid rate, and crops are harvested each year, you would probably have to apply virus each season and maybe several times during the season. There are other systems, however (for example, in a forest environment), in which you could introduce the virus and thus induce an epizootic which could prevent damage by the insect.

FALCON: I would like to make comments regarding quality control. One can have all the necessary quality control in developing a commercial product, but yet, when it comes time to apply that product all can be lost. Today most of the pesticide application work around the world necessitates the use of water. Water may be used in small quantities, of say 5 gallons or less per acre, or as much as 800 to 1,200 gallons per acre for application of a pesticide. The source of the water is important as is its quality. In some situations water is simply pumped from a local stream or a swamp, and who is to know what is in the water? So you see, quality control extends far beyond the product.

With regard to the questions of why the viruses have not taken over and if they are so specific, why are chemical companies having so many problems? Unfortunately, in our situation, and with the kinds of games we play, we are continually asking these same questions of ourselves. Some factors include the existence of our free enterprise system and the fact that the chemical industry has a considerable amount of resources because it is selling products that are producing profits based on royalties. They engage in heavy advertising, a high degree of merchandising, and personal customer service. It is a very difficult system to penetrate. The system has developed over time, and it is a way of life that the farmer and other users of pesticides are accustomed to. On the other hand, when dealing with baculoviruses or other biological control agents that have little or no patent possibilities, there is little money involved, advertising is limited, and there is little publicity. You seldom see billboards saying, "Use parasite A or Pathogen S. It is good for you, it will save you money!" There are a few publications, such as Organic Gardening, that expose this kind of thing, but generally, it is very, very limited. So in a free enterprise system where merchandising and patent rights and royalties are the dominant features, the kinds of things we are trying to do are very difficult.

STOLLAR: Dr. Fields will now discuss certain aspects of the genetics of viruses, and how these may relate to the problems we are discussing today.

FIELDS: I am a geneticist and I should describe what I heard, and what I have heard so far is about viruses that have a very specific host range and a very specific ability to replicate that is safe, and certainly, everything that we have heard suggests that is true. The reason I think we are here, in part, is to ask if this is true both for the viruses that have been developed, for others that will be developed, and also, will it always be true, or is it true to a certain finite point, or is there a statistical or biologic reality that there may be a risk beyond what we are aware of? Really, what I am saying is not, are these viruses safe and stable, but how do they change? That is, do they change? How do they change? And what are the strategies that viruses use to either remain the same or become different?

I am putting it into a theoretical framework, and I would like to come back to the practical questions as I see them in terms of some points that I would like to recommend as a geneticist. First, I would like to briefly outline how all viruses seem to change and describe their general strategies. Then, I will outline some of the ways that other animal viruses in the last few years have been shown to use strategies to change. I think it will be clear that some viruses change often and easily, and therefore, are very difficult to eliminate -- think of influenza -- and other viruses tend to be much more stable and have fewer opportunities for change, such as polio virus, for example. I would then like to focus on some genetic issues that I have heard of in my very short contact with this type of virus.

The first point is general strategies for change. All viruses and all living creatures either can mutate or recombine, and mutation can involve anything from point mutations to frameshift mutations -- in more complex creatures with chromosomes, to actual loss of chromosomes, deletions, duplications, etc. Recombination can either be classic recombination with breakage and reunion of DNA, or copy choice mechanism, or can occur by mechanism of reassortment; that is, by exchange of segments rather than a breaking and reunion. The difference is that mutation is actual creation of new genes and recombination is shuffling around of old sets of genes.

I will briefly outline some major examples from animal virology that parallel the viruses we've been hearing about. First, there are herpes and adenoviruses (which are large DNA viruses), which have been clearly shown in recent years to undergo recombination, and in many ways these are the viruses that will have to be most closely modeled. Second, there are viruses such as influenza, reovirus, and the bunyaviruses (which have segmented genomes), which have been shown to use two primary mechanisms -- one is that all of these viruses have been shown to various degrees to undergo very efficient reassortments. In addition, at least reovirus and flu viruses undergo mutation as well. For example, influenza virus, although not absolutely proven, very likely derives the new segments cross-species; that is, when a new virus comes in, even though influenza is a human virus, it is very likely that new genes come from other species. Even though swine flu did not occur this year, pigs, avian species, and other nonhuman hosts can act as sources for new genes -- which I think is going to become at least something to strongly consider when some of the segmented insect viruses as genetic systems are developed for them. The term antigenic shift refers to insertion of new genes, and antigenic drift refers to the mutational changes that cause less dramatic changes in the protein.

The third type of virus that is relevant to the small RNA linear viruses, such as polio virus, is somewhat controversial. There is evidence suggesting that they may undergo recombination, but if so, it occurs at a very low frequency, and it is quite likely that they undergo mutational changes as part of their way of changing. Certainly for other linear RNA viruses, data for true recombination, in the sense of DNA, have been very difficult to get. This system is obviously going to be applicable to that other group of picorna-insect viruses.

Now in addition to interactions between two parents of the same type of virus, a second type of genetic interaction that will have to be considered in insects is between a virus and an unrelated virus. For example, it is possible to derive recombinants between adenovirus and SV40 virus, and, in fact, these viruses were recognized as contaminating the early polio vaccines. The adeno-SV40 hybrid virus is then a recombinant between two viruses that are very different.

These types of genetic interactions that I have just described are genetic in the sense that the genes interact directly. There are two relevant interactions that involve gene products that I think are good examples. One is the formation of pseudotypes. A pseudotype is a virus that has the nucleic acid of one virus coated in the protein shell of the outercapsid of a second type of virus. And there are now numerous examples of this type of interaction -- most recently many examples studied involving vesicular stomatitis virus, a rhabdovirus, similar in structure to some of the viruses we have seen, and leukemia viruses. This is important because it can alter the host range.

Another type of genetic interaction that seemed to stand out in the presentations this morning was complementation possibly occurring between particles that have multiple genomes enclosed within a single coat. That is what, in the case of paramyxovirus, we would call polyploid or heterozygous particles that have numerous copies of the same genome. These particles can interact by having the gene product of one defective member of this mixture interact with the second and change the biologic properties of perhaps the first. I would like to point out that the polyploidy is certainly a very general phenomenon among certain types of viruses, and paramyxoviruses offer some very interesting parallels, particularly with some studies involving the Newcastle disease virus.

Before briefly outlining impact, I would like to point out that host genetics become very important to discussions of viruses that are affecting cells and hosts, and the possibility of host-range mutants exists. It is also possible to take the same virus and get different results in perhaps different cells in the same animal -- either cell culture system or insect. This is a very common finding if one takes one virus and infects a cell population, and then clones the cells that result. One can see a variety of biologic effects of such a system, even from a single cloned virus. There are examples of this in SV40.

In conclusion, the ultimate impact that we are concerned about is host range and virulence. The questions that we are really asking are: What is the degree of variability, what are the options of the viruses, and how stable are these in terms of population dynamics, in terms of host range, and in terms, ultimately, of virulence? We know that mutations occur in virtually any large population of viruses and living creatures. We know that interactions can occur between viruses, and it seems to me that the genetic systems that are going to have to be developed are going to have to ask and test concerning the broadness of some of these interactions and the frequency of mutations by different agents.

Again, I would like to emphasize the fact that one of the reasons the influenza virus is still with us is because it knows how to vary. It varies both in the individual genes coating for the two capsid proteins, as well as by the introduction of new genes. Thus, it is an extraordinarily interesting and variable virus that tells us of the potential for change. We don't really know this yet about any of the insect viruses; moreover, it will become very important, particularly when one talks about such creatures as the cytoplasmic polyhedrosis viruses with segmented genomes, which are not totally relevant here but have been used, as well as the Nodamura virus with a segmented RNA genome. Host range and virulence can be altered by a mutation recombinational mechanism.

Now the practical versus real summary in this issue is that the use of chemical insecticides coupled with viruses is a very novel approach which will raise questions to geneticists. I would like to know whether these are mutagens because by coupling a chemical insecticide with a virus, one is, in fact, asking to increase the frequency of mutation and to increase what may in fact be stable under certain circumstances, to an unstable state. It is very important to know whether chemical mutagens will have mutagenic properties in agents that at any point may be co-circulating in the population.

I would like to make the following concluding points. First of all, from everything that I've heard, genetics work is presently highly feasible for these viruses. Not only is it feasible, but I personally think it is quite urgent to develop genetic systems in order to begin to have the right framework to know what questions to ask. The cloning of the virus is feasible. Cell culture systems are available along with single cycle experiments for their study. Host range sounds like it may be available, or certainly the approach is reasonable, and there are numerous biochemical markers that serve as superb markers for genetics that have been identified by several of the presentations. It seems that the genetics of at least selected members of these viruses would be highly worthwhile to support.

IGNOFFO: There might have been a misinterpretation when I was talking about chemical pesticides. What I was trying to relate is that chemical pesticides normally are not used together with virals in an entire system approach. What the integrated pest management type system should not use are chemical insecticides, except as a last resort. We know that some of these chemical insecticides are mutagens.

FIELDS: In some of the discussions earlier, the issue of having either a chemical pesticide in the population at the same time or possibly combining the two in a more potent combination therapy has been raised. I personally would urge caution in terms of the potential mutagenic capability of that approach.

FAULKNER: You were asking, do viruses change? We've been quite interested in this sort of thing, and we worked with plaque-purified strains of nucleopolyhedrosis virus of _T. ni_. We tried to find out what happened when we passed them serially _in vitro_ and _in vivo_. And, as you pointed out, we have to ask which are to be used. For the _in vitro_ passages, we've tended to use phenotypic markers, such as what we call many polyhedra and the few polyhedra produced per cell. We find the insect tissue culture system that we work with tends to fade with the production of the few polyhedra type of strain of virus, which can be plaque-purified and remains FP, as we call it. This is fairly well established.

We've also run a series of studies where we've picked plaque-purified many polyhedra virus; that is, the most virulent virus. These plaques were picked for the highest virulence that we could obtain and we've serially passed these in the homologous insect -- Trichoplusia ni. We initially started with plaque-purified virus. We harvested the polyhedra from insects four and seven days post infection and then continued that series to see what happened to virulence. We continued that for 16 passages and found that there is no significant change in virulence as far as that particular marker is concerned, having started off with an MP type of virus. We examined the hemolymph with each stage of this to see if there were a phenotypic variation; we did not find it. These viruses have been with us a very long time, but the virus and the host, in this particular instance, have brought themselves together pretty well, and we are actually working with probably the best of the virus.

The other point that I would like to make is that you've said we are at the stage now where we can look at homologous and heterologous virus interaction, and so on. I think that a number of people are very interested in this.

Further, I am quite concerned about the people who produce the viral insecticides commercially because they tend to produce their inocula from previously killed populations. Now from what I've told you, there probably is no major change in virulence, but that seems quite unmicrobiological. With several of the viruses that we are talking about, we could be producing the seed inocula in tissue culture and proceed from there.

SUMMERS: I'd like to refocus attention here for just a minute -- let me remind you that our present plaque assay is a polyhedral plaque assay. We've identified several problems associated with this today in terms of whether or not polyhedrin synthesis or polyhedra is a good and/or reliable marker. It is within those systems that we have characterized and standardized, and we know how TN368 cells respond to a given multiplicity of infection, etc. However, I think if we are going to address ourselves to the very problems that you have summarized, we have to do so with marker systems specific for and at the level of replicating virus. That is, the enveloped nucleocapsid, and not some gene product of viral infection such as polyhedrin, which we've already seen can be influenced by the cell, by the environment, perhaps by the virus, even though it is standardized and reproducible in a given system. I think this is one of our major problems in screening -- monitoring and detecting change. We are not quite there yet, and I would like to see us reemphasize and focus on the need for those kinds of studies.

FIELDS: I would agree. Also, I think it is very crucial to distinguish what is phenotypic (something you are scoring as a phenotype) from a direct assay for the genetic event (the genotype), and whether it is a biochemical marker for a segment of nucleic acid, or a restriction cut that shows segregation for a particular marker. The ways to test whether that is a phenotype or a genotype would be to use genetics, isolate mutants by traditional means, and see if there is segregation for polyhedra for a particular region of the genome.

SUMMERS: I would like to emphasize further that we need to take a comprehensive approach to this, which involves the restriction endonuclease mapping, as well as looking at virus-specific proteins or developing plaque assay systems for the replicating virus. I don't think the focus here today, although it has emphasized serology and basic studies on virus structural polypeptides, is at all excluding direct analysis on the viral DNAs themselves.

ZAITLIN: I would like to reinforce what Dr. Fields has said with respect to strategies for genetic change. And I will give as an example some plant virus work. Of course, it is appreciated that there are arthropod-transmitted plant viruses which are, of course, very important in nature. There are examples of what we would term transcapsidation, rather than the formation of pseudotypes, which tend to extend the host ranges of virus, or at least can in theory. For example, the barley yellow dwarf virus is rather specific in that there are a number of species of aphids and a number of isolates of the virus. The specificity is very great, but in circumstances involving a mixedly infected plant, it is possible to get the wrong aphid transmitting the right virus, which is an example of transcapsidation. This virus, of course, has the wrong protein coat on it. Then you can visualize the aphid proceeding to some other species of plant that would not normally host this virus, but it would then get the virus and the virus could not, theoretically, replicate in that plant.

IGNOFFO: Is there any way you can relate to us the frequency of drift or shift in the influenza virus, that they could actually measure and determine that there is a shift in comparing passage through cells and through an *in vivo* system in man?

FIELDS: I think that is a very important question. I am not talking about passing this necessarily once through man. First of all, the origin of that example is not known with certainty, that is, exactly where the new genes come from. There are hypotheses. But it is very clear that every 10 to 15 years, a new gene is introduced, that is the antigenic shift, and that correlates with pandemic flu influenza. That is the virus as it infects people, and it has been shown experimentally that you can, in fact, create new viruses by co-infecting pigs, for example, with different strains. You can get recombinants between human and pig strains. The concern is -- do we know enough, with any virus, when we are putting a segmented virus into population, when one has other viruses circulating and one has latent viruses -- to know that the opportunities under unusual circumstances for introducing new genes will not exist. In fact, one of the most crucial questions with flu, reo, and with other segmented viruses is where, in fact, does this occur? We know it can happen and that it correlates with epidemics. The question here would be, if you have circulating segmented viruses, can they interact? I think that is a research question. What is the range of reaction between cytoplasmic polyhedrosis viruses? And then in getting interreactions, do you change the host range of these viruses, such that if you used one as a pesticide, would you really be as specific as you thought?

(Inaudible comments from the audience.)

This is very high frequency in both systems. That is, the frequency of reassortment is an experiment that has been done both in cell culture systems and has been done directly by putting influenza virus into the lungs of pigs (into the respiratory tract), and it's efficient enough so that a limited number of animals, when infected with both parents, will come out with recombinants. So it is an extremely efficient phenomenon, and I cannot say for sure this is happening with the segmented insect viruses, but it's unlikely that it's not.

SUMMERS: I think we should go further and say that in the case of baculoviruses, or other insect viruses, that this need not be considered a detrimental or an undesirable property, because some people immediately extrapolate that this is akin to mutation to some other terrible form. This in fact is natural. What we need with baculoviruses are systems to monitor these changes and to begin to develop a data base so that when necessary we then can discuss with specific examples and data an understanding of mutation, frequency change, antigenic shift, etc.

FIELDS: I think it is absolutely important to emphasize that. We ask these questions in order to get the data, not to say that because something could happen under conditions that are perhaps artificial or unrealistic, one should not get the information. I think that is a very key point that I agree with.

HOLOWCZAK: First of all, I would like to say that I've been impressed today by the presentations of some very elegant data studies, and I think the systems are very difficult. I also believe there are a lot of good data resulting from these studies. In general, I want to say that what is being said here by people who are experienced with their vertebrate models is not being said because we are endowed with some special knowledge, but it is because vertebrate virology is a little bit older than insect virology, and we virologists have made a lot of mistakes. Well, in the sense of having a good tissue culture system in the study of virus, I think invertebrate virologists have made a lot of mistakes. I think what we are trying to say is, don't make the same mistakes we did. There are a lot of problems. I recall particularly the experience with trying to control rabbits in Australia using a vertebrate virus, which essentially failed.

The other possibility is that of developing resistance to the viruses that you are using. Perhaps someone will address that issue.

I heard Dr. Tinsley speak recently, and he tells me there are no new ideas for the development of new insecticides. People are pretty well exhausted in the area of new chemical development; so therefore, we may be forced ultimately to go to insect control. And, if when we have insects that are already resistant to the known chemical insecticides we then develop resistance to viruses, what is left? I think this is something to consider; perhaps we should be working on a third control mechanism of chemicals and viruses, which may be important.

The other thing I think which is intrinsic in what is being said here, is the fact that it has taken a long time for animal virologists to realize the different modes of interaction of viruses with cells. The fact that we cannot only have cell killing, but we can go to the transformation of cells that live and grow; we don't necessarily have to kill a cell. Most animal virologists don't understand what causes CPE in animal cells; we've been looking at it much longer, perhaps been looking at insect cells, and we are not really too much ahead in that sense. There are, nevertheless, situations like slow viruses where you introduce the virus, and it takes many, many years before you see the effects of that virus. And that is something that concerns everyone, I think.

The other point is that I have attended four or five seminars now on insect viruses, and one story that is always told is how much virus one can get from eating coleslaw. Repeated in many of the conference reprints is the quote, "You can get X number of milligrams of occluded virus by eating one good helping of coleslaw." We could say, "So what difference does it make if we spray with these viruses because it is all there naturally, and we are going to get a huge dose anyway." That really concerns me because, as it has been pointed out, the use of these viruses is very limited, and yet they are already present in nature and we are already eating them. If we begin to spray extensively, the question is, how much will we be getting at that point? As someone pointed out to me, it is difficult to tell because of the social changes and organic food people who won't wash their food, much less cook it, we will probably have a problem with them, because they will be eating an awful lot of virus. Problems like that arise. So the question is, what will be the antigenic dose that will be permissible in our population?

Another thing, people are going to develop all kinds of hypersensitivities, which can be very bad in many ways so to speak. The final thing is that I am continually struck with the similarity between the entomopox viruses, which reportedly infect only insects, and those pox viruses that infect mammalian cells. One looks at the virus, DNA is the same size, and they contain the same enzymes needed for the replication. They seem to replicate in the same place in the cell, and the question that emerges is: How is a mammalian virus different from an insect virus?

I think that's a very interesting question. One thing, I was struck by the fact that vaccinia virus and the pox viruses in general tend to cause a delayed sensitivity type of reaction after they are injected into humans and cause T-cells to activate. I wonder if anyone, for example, has looked at this phenomenon in a rabbit. Using an entomopox virus that you might have -- what would interest me would be to have a nice antigen that doesn't replicate, hopefully, but would induce T-cell activation because it might be a very powerful tool to study this phenomenon. But it still bothers me that these viruses are so similar, and yet they are apparently restricted in their host range so very tightly, and one wonders if this won't eventually break down in some way. There will be insect viruses that will affect mammalian cells.

COLLINS: I'd like to follow up on one point that may also be relevant for tomorrow's discussion about safety. But obviously, one of the things that we are worrying about is what effect these viruses may have on humans that come into contact with them, if they are used as pesticides. It seems that we have a very good captive population right now with people who are working with these viruses in terms of what sort of an immune response do laboratory workers who work on these viruses rate. I talked with Dr. Harrap earlier and I think his experience is rather unusual in terms of the type of pattern with antibody response that he has seen. But I am curious about the experiences of other labs -- whether they screen personnel and themselves for antibodies to these viruses -- and what sort of antibodies are raised. Obviously, it is very early in the game to know whether they are group-specific or type-specific, and this is really just a starting stage, but I think this would be a very good population to look at in this regard.

HARRAP: We started screening our staff fairly routinely some years ago, and initially, we could pick up reactions to baculoviruses to the inclusion body protein as an antigen in a fairly small percentage. It was no more than 10 percent of the personnel in the lab. We do this roughly every six months, and in the last two screenings that were done, the staff exhibited no reactions to baculovirus inclusion body protein.

IGNOFFO: This is routinely done in commercial production, and it has been done for a while. I am recalling one of the first studies of the screening, which was done in Japan by Dr. Aizawa on people handling silkworm NPV. There continues to be regular monitoring for clinical serology and tests for infectivity using the neonatal system (which employs the youngest larvae possible). All tests have been negative.

SMITH: I think there are a couple of things we can look at in the context of history that really should be considered, and perhaps this is an appropriate time for this. In the case of the myxomatosis viruses that have been used for control in Australia and so on, it is really quite clear that two organisms are involved. There is the virus and there is the host, and one of the things that animal virologists have envied about the insect system is the genetics that are already available with the host, specifically, *Drosophila*. It seems inconceivable to me that this experiment hasn't been done. Perhaps it has. You simply take a large population of Drosophila and expose it to a small or even a large population of one of the virus pathogens that is pathogenic.

(Tape change.)

What other kinds of biological nonchemical treatments are available? Specifically, a few years ago there were some attempts to use a bacterial agent that was infectious for insects.

IGNOFFO: Bacillus thuringiensis has been on the market since 1959. At present, it probably has sales in the United States of about a million and a half pounds per year, plus or minus 10%. There are other microbial agents also. There is Bacillus popilliae (a bacterium of the Japanese beetle). There are fungi, which have been developed in other countries. Protozoans are also being developed. So, all typical groups of pathogens have been looked into as potential "microbial insecticides."

FALCON: There are many examples in the literature especially in the first part of this century involving the utilization of different forms of biological and natural controls. Today this is also true in the types of programs we are attempting to develop that are termed integrated control or integrated pest management. In these programs the objective is first to use as much as possible naturally occurring biological controls, and second, to manipulate them or to somehow increase and enhance them, etc. For example, there are privately owned operations in the United States that produce and sell living insects that are used for biological control. Then there are examples of pathogens, some of which we have today. Also more recently, insect hormone and pheromone research has occurred. There is now a commercial product containing a synthesized insect juvenile hormone that is used to control mosquito larvae. Thus, there are many possibilities of interesting and useful substances that may be combined for pest control purposes. However, there are many problems associated with their development. There is the problem of registration, and this immediately creates problems for development because to put together an insecticide that contains several pathogenic organisms requires first that each pathogen be registered independently. For each one, safety, efficacy, and usefulness must be demonstrated. Only after this can one one begin to talk about a package approach. Simply registering one of these materials today is very costly. As I pointed out earlier, private industry is not motivated to do it, and until society, the government, and we are willing to provide funds for that type of research, the activity will continue to be very minimal. But certainly your suggestion to develop polyvalent types of viruses is a very good possibility. Perhaps within two or three decades, we will be using polyvalent types of viruses instead of chemical insecticides.

HOOD: Although I am not a virologist, as a spectator of today's seminar and an employee of EPA who is worried about registration, I would like to say that it has been a very good day. One of the things I would like to hear discussed even a few months from now is how EPA should deal with the capacity for change possessed by these viruses. If, after a particular virus is registered with certain qualities, it later invokes a large capacity for change and begins to do something it did not do originally, a myriad of problems could develop. This capacity to change may be innocuous enough, it may not hurt anything, but on the other hand, if it provides a threat to a pollinator group, or a threat to mammals or people, then how do we institutionally cope with this? What should we look for? What are the signposts in a research program that we should find? What is it going to cost? In which direction should we go?

There was one other comment about the similarities between insect viruses -- I think it's a pox virus -- and animal viruses. I think this is the kind of thing that is really kind of frightening when we are going great guns and all of a sudden it jumps the track and we have released a large amount of viruses with a great capacity to change. I would like to hear some discussion on this.

FIELDS: I think it is important to comment on this as I think one of the attitudes one comes in with is to ask questions, some of which, in fact, are quite unlikely to be directly relevant to what is going on. For example, genetically, I think it's fair to say that every living creature has some intrinsic ability to change, i.e., via mutation; there is a background of mutation rate, there are opportunities for recombination. Now that does not say, in fact, that this is directly practical or directly relevant to what is going on. In fact, questions sometimes can be answered only after many years and often the answer will be exactly what you thought it was in the beginning. The real issue in raising the question of genetics is the capability, the feasibility for asking such questions about mutation rate and gene structure. Strategies of insect viruses now exist so that you can really begin to learn which are the right questions. The fact that something can change doesn't mean that this is practically important or that years of experience with something that show it to be safe should be curtailed necessarily. For example, just because it is possible that that might change, and we are all living in an era of heightened awareness of unusual happenings, it is very easy to create a scenario that would scare us and almost destroy the ability to do anything.

HOOD: I think what I am looking for is, how do we assess this change? You said some viruses change very rapidly and some are quite stable. I think what we really need to have is a set of guidelines or criteria, because, obviously, we need to sort out the stable from the unstable. It's always going to change, but I guess sometimes we need help on formulating what do we do next.

FIELDS: Basically what you have done is ask a question that, until the focusing on genetics had become feasible, one would not know how to ask. That is, until you know the gene organization, the gene structure, mutation frequency, and the general strategies, the question cannot in any way be asked to provoke a good answer. What we both are saying, I think, is that we need to know more about the genetics of these viruses; then we can begin to know the right way to ask proper questions. I think that really is the crucial lesson: not to make, for example, the parallel that I made for demonstration purposes of influenza, which is an unusual virus, and not to translate that into insect viruses as they currently exist, but to translate that into the need to develop a real information base using a few model systems, so that we can perhaps answer that question a few years from now.

IGNOFFO: The point that Dr. Fields is making is a very good one, because the kind of questions that you can ask can't be placed in a theoretical or a meaningful frame. Do we have the techniques to answer those kinds of questions? I don't think we have for most of these right now. But they can be developed along the classical lines that have been developed from mammalian virology.

ZAITLIN: I think in one case we do have the tools to test this. There is an example given with respect to the similarity between vaccinia virus and some pox viruses of insects. I wonder if this is really an example of convergent evolution of viruses that, in fact, aren't really related. Dr. Fields himself gave the example of SV40 and adenovirus, which are very similar, although they have differences. If you try to do molecular hybridization between the DNAs of those viruses, they won't hybridize, and I can give an example from our own studies. We work with several strains of tobacco mosaic virus, and by all criteria you can judge these to be strains of the same virus, yet when you do molecular hybridization, they do not hybridize at all. It turns out that these are viruses originally isolated from quite diverse species of plants, and I think it is an example of convergent evolution. I think we could test these sorts of things, at least with some of these marked similarities between the mammalian and the insect viruses.

PART V
SAFETY: A CRITIQUE

Review of Safety Tests and Methods of Evaluating Infectivity

Clinton Y. Kawanishi, Ph.D.
Environmental Protection Agency
Research Triangle Park, North Carolina

INTRODUCTION

Viral pesticides can be effective alternatives for problem or inefficacious chemical pesticides under certain circumstances. To register a pesticide with the EPA, the formulation must be evaluated for hazard according to certain specified guidelines (see Federal Register 40, pg. 26831, June 1975). The data for a viral pesticide must show that it is not a pathogen of man, other vertebrates, or nontarget invertebrates. The required tests are presented in the Guidance for Safety Testing of Baculoviruses. It must be emphasized that it is the formulated product, not the active ingredient, that is registered for use with the EPA.

Presently, there are two registered baculovirus pesticides; ELCAR® for control of the cotton bollworm, *Heliothis zea*, and the tobacco budworm, *Heliothis virescens*, on cotton; and TM Biocontrol-1 for control of the Douglas fir tussock moth, *Orgyia pseudotsugata*, by the U.S. Forest Service (Federal Register 41, pg. 51067, 1976). Many other baculoviruses are presently being tested for their safety to man and the floral and faunal components of the environment.

Much of what I present here has appeared elsewhere (1, 2, 3). Some of the information was presented at the EPA-USDA working symposium on Baculoviruses for Insect Pest Control: Safety Considerations (4).

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IN VIVO SAFETY STUDIES

Extensive in vivo tests have been conducted to assess toxicity-pathogenicity, allergenicity, teratogenicity, and carcinogenicity of invertebrate viruses to vertebrate species (1, 2, 5). For a partial list of some of the viruses tested see Ignoffo (1, Table 8). The largest number of tests were done with the baculoviruses, especially the H. zea nuclear polyhedrosis virus (NPV), the active ingredient of ELCAR®.

The range of doses administered in the safety tests of H. zea NPV, as an example, are shown by Ignoffo (1, Table 4). The number of polyhedra applied ranged between 1×10^7 to 3×10^{12} per kg body weight of the test animal. In addition, virus released by alkali from polyhedra, virus from the hemolymph of infected insects, polyhedrins, and virus DNA were tested. The species used for the evaluations are listed in Ignoffo (1, 2, Tables 9 and 3, respectively). Tests were performed on rodents, dogs, birds, fishes, mule deer, and primates, including man. Cross-infectivity studies among invertebrates species have also been done (6).

Viral inoculation was done by peroral intubation; diet feeding; dermal and eye application; intradermal, intramuscular, intracranial, and intravenous injections; and by inhalation. In none of the studies were acute symptoms or other indications of baculovirus infection encountered. The parameters monitored or evaluated in these studies were most commonly: weight gains, body temperature, external symptoms of infection, activity changes, necropsy with examination for gross pathology, and histopathology of selected tissues. For sensitization studies, wheal formation and size, as well as other dermal manifestations were checked. Tests for antibodies to the virus administered, observations for polyhedra in test animal excrement, and bioassay for infectivity of selected tissue homogenates were performed in some cases.

Aside from the baculoviruses, similar experiments were conducted on mice, rats, and rabbits with some entomopoxviruses and the parvovirus of Galleria mellonella, the densonucleosis virus.

A picornavirus, Nodamura virus, which was isolated from apparently healthy Aedes tritaenorhynchus in Japan, caused illness with hind limb paralysis seven to ten days after it was injected intracranially or subcutaneously into three-day-old mice (7). Evidence also showed that this virus multiplies in ticks and Indian meal moths. Bailey and Scott reported on the cultivation of Nodamura virus in the honey bee and greater wax moth (8).

HUMAN TESTS AND MONITORING OF EXPOSED PERSONNEL

Tests were conducted with the *H. zea* NPV in humans (9). For five days volunteers orally ingested 1.2×10^9 polyhedra in gelatin capsules. Subjects were given complete physical examinations prior to the tests and nine and then thirty days after the tests began. Ophthalmoscopy, examinations of the skin and mucous membranes, percussion and auscultation of the chest, palpations of the abdomen, search for enlarged lymph nodes, and examination of the extremities were part of the physical. Weight, temperature, blood pressure, pulse rate, and respiration were also noted. Urinalysis, hemoglobin level, hematocrit, differential white cell counts, and differential cell counts, serum glutamic oxaloacetic transaminase, thymol turbidity, cephalin flocculation, alkaline phosphatase level were determined. As before, these studies revealed no evidence of viral infection.

Human exposure to the *H. zea* NPV during manufacture of the viral pesticide was also monitored (10). The general health of the individuals was checked and urine and blood samples were also tested. Urine samples were bioassayed against neonate larvae of *H. zea* for infectivity. Sera were tested for the presence of antigens of intact polyhedra by Ouchterlony immunodiffusion, the presence of antibodies to partially purified NPV virions, and hemagglutination titer. No allergic response was noted except for one case of aggravation of a preexisting asthmatic condition. Clinical symptomatology or immune response indicating subclinical infection was not detected. Nor was there any indication of viral material in the blood according to the tests applied.

Serological studies and survey of laboratory workers exposed to insect viruses were reported by Tinsley and Harrap (11). None of the personnel exhibited any symptoms of viral infection from material handled in the laboratory. Sera were tested against an insect parvovirus, an iridovirus, a granulosis virus, polyhedrin from an NPV, and a small RNA virus. Antibodies of two individuals against the picornavirus were detected.

IN VITRO STUDIES WITH INSECT VIRUSES

Although they are increasing, the number of *in vitro* studies dealing with infectivity of insect pathogenic viruses to vertebrate cells are still relatively few. Some of the cell types previously tested are shown in Ignoffo (1, Table 11). Unlike the situation with *in vivo* studies, some positive tests have been reported. The majority of reported infections of vertebrate cells in vitro used viruses that have been grouped in the past by invertebrate virologists, often for the sake of convenience, into an artificial category referred to as nonoccluded or noninclusion viruses. As you are well aware, this encompasses most of the known animal and plant viruses.

The death of some chick embryos after inoculation with a baculovirus, the NPV of the silkworm, Bombyx mori, was reported (12). Chorioallantoic membrane was inoculated with 3000 rpm supernatant of infected silkworm blood. Virus could be detected by bioassay in silkworm pupae only up to the second passage. Because no appropriate controls were described, the effects cannot be definitely attributed to the virus itself. The author also inferred changes in the morphology of the polyhedra after alternate passage in chick embryo and insect pupa. Attempts with turkey eggs using alkali released particles and polyhedra of the nuclear and cytoplasmic polyhedroses viruses of the silkworm by Cantwell et al. (13) showed no increase in the rate of parthenogenesis over controls. Polyhedra were not present in histopathological preparations of parthenogenetic undifferentiated embryonic membranes or in the embryonic fluid or membranes after injection.

More recently inoculation of WI-38, primary monkey, porcine and bovine kidney cultures with purified virions, hemolymph from infected hosts, and DNA of the NPV of Choristoneura fumiferana, the spruce budworm (14), showed no cytopathic effects (CPE) attributable to the virus. The uninfected hemolymph of the host, however, proved to cause CPE in the cultures of these vertebrate cells. Inoculated cultures were carried through three passages at 28°C or 37°C before the experiment was terminated. Cells were carried through only a single passage in the attempted transfection.

Banowetz et al. (15) reported no pathological changes observable with the light microscope after five passages of Salmonid cell lines, CHSE-214 and STE-137, treated with infectious hemolymph from Douglas fir tussock moth NPV infected larvae. Electron microscopic examination of cells, 24 hours post-inoculation, for viral infection did not reveal any indication of virus. Growth rates did not differ between treated and untreated cell cultures.

Preparations of alkali-released virions of H. zea NPV, when used to inoculate African green monkey kidney, human primary embryonic kidney, HeLa, and WI-38 cells produced no CPE after three passages at 37°C. No hemadsorption of guinea pig erythrocytes or interference with ECHO 11 infection was observed (16).

Studies by McIntosh and colleagues indicate association of NPV with vertebrate cells. Using polyhedra and alkali released particles of *H. zea* NPV, they inoculated cultures of primary human amnion, human foreskin, human embryo, WI-38, and leukocytes (17). No CPE was detected over a 4-week period, and viability of inoculated and uninoculated cultures were similar. Treatment with *H. zea* NPV did not increase DNA synthesis in leukocyte cultures. No transformed foci in primary human embryo cultures occurred, and no evidence for leukocyte proliferation in response to viral inoculation was noted. When inoculated cultures were assayed for infectivity to cotton bollworm, however, virus was shown to persist in these cultures up to 4 weeks. However, immunofluorescence studies with conjugated antibodies to alkali liberated virus failed to detect virus in primary human amnion cells. NPV inoculation did not enhance or inhibit SV40 infection of PHA cells.

In a similar study (18) which used immunofluorescence and autoradiographic criteria, the effects of *Autographa californica* NPV on a poikilothermic vertebrate cell line were assessed. These viper cells, VSW, which were inoculated with [^{3}H]thymidine-labeled virus exhibited grains mainly distributed over nuclei. Positive immunofluorescence with conjugated antibodies to "infectious hemolymph taken from *Trichoplusia ni*" was reported. The fluorescence was prevented by prior treatment with unconjugated antiserum. The authors interpreted these findings as evidence that *A. californica* NPV became associated with VSW cells. Granados (19) has electron microscopic evidence of HeLa and FHM cells uptake of *A. californica* NPV in vacuoles and retention of virus in these structures in undegraded form.

The one successful, but unconfirmed, report of transfection of Fogh-Lund human amnion cell (FL) with the silkworm NPV-DNA (20) utilized the hypertonic 2.2 M MgSO$_4$ method (21) at pH 7.4. Nuclear inclusions of characteristic polyhedra shape appeared in a small number of cells without CPE after 13 days. These bodies appeared only in cultures inoculated with NPV-DNA and NPV-DNA treated with RNase. Comparable FL cells inoculated with DNase digested NPV-DNA, calf thymus DNA, virions, or Tris-HCl buffer showed no such structures. The inclusions were dissolved by Na$_2$CO$_3$; were resistant to trypsin like polyhedra; were not lipid in nature as they were unstained with Sudan black; and unlike nucleoli and other normal nuclear components they were only weakly stained by Giemsa. The alkali-dissolved inclusions gave a positive ring test with antinuclear polyhedra serum and were infectious to silkworm pupae. However, the results should be viewed with caution because of the small number of animals used in this bioassay. This represents the only report of baculovirus replication in vertebrate cells.

Cell culture studies with other insect viruses have been reported. A member of Reoviridae, the cytoplasmic polyhedrosis virus of Trichoplusia ni, the cabbage looper, was tested for its infectivity to HeLa, L, and four mosquito cell lines. Cultures of these cell lines and TN368, a susceptible cell line derived from the cabbage looper, were inoculated with homogenates of CPV-infected T. ni midguts. No evidence of viral infectivity was observed by phase or electron microscopy, except in the chick TN368 cells (22).

Roberts and Campbell (23) reported that two entomopoxviruses enhanced exogenous fusion when virions were centrifuged at 1,000 x g onto L, HeLa, and BHK-21 cells. At a multiplicity of 100 virions per cell, the extent of cell fusion was directly related to centrifugation time. The polykaryocytes increased up to three hours and began to disintegrate after 20 hours. Remaining uninuclear cells started to divide at 30-36 hours and reached precentrifugation numbers at 96 hours. Bioassays indicated most virions were not cell associated. Fat head minnow and 3T3 cultures were not affected but cells of five mosquito cell lines and TN368 suffered cell death and lysis without fusion. BTI-EAA cultures, which support the replication of one of the insect pox viruses, did not fuse or lyse.

The entomopox viruses also interfered with Vaccinia plaque formation. Multiplicities of 100 and 1,000 virions/cell reduced plaques by 50% and 90-99%, respectively. Interference was increased by trypsinization of virions, reduced by UV, and eliminated by heating at 56°C for 30 minutes. Experiments indicated that the interference was not due to infectivity but to host cell impairment and a normal low level of cell fusion.

Chilo iridescent virus (CIV), a member of the Iridoviridae, was shown to be capable of infecting viper (VSW) cells (24). Evidence indicated an increase in viral titer, cytoplasmic DNA synthesis after virus inoculation, positive immunofluorescence for viral antigen at 24 and 48 hours after inoculation and EM evidence of cytoplasmic viral assays, and particles with CIV morphology budding from the cells.

Kolomiets and Alekseenko (25) isolated a picornavirus-like 30-70 nm isometric virus (probably acute bee paralysis virus) from infected hives. They propagated the virus in 10- to 11-day-old chick embryos by inoculating filtrates of dead bees into the chorioallantoic membrane. Pathological changes in the chorioallantoic membrane and embryos with hyperemia in the region of the head and neck were frequently observed. The authors were able to reproduce typical clinical symptoms when pathological material was injected into healthy bees. This virus hemagglutinated rooster erythrocytes and a hemagglutination-inhibition test and immunodiffusion test for virus were developed.

Nodamura virus, a small RNA virus isolated from inapparent infections of mosquito, multiplies in BHK and mosquito cell lines without CPE (26).

An invertebrate parvovirus, the *Galleria mellonella* densonucleosis virus (DNV), has been reported to adapt to mouse L cell line (27). Infected cells showed fuelgen positive intranuclear inclusions three to four days after inoculation, positive nuclear fluorescence, and electron microscopic observation showed particles in L cells. L cells and rat embryo fibroblasts culture are reported to form foci of transformation by densonucleosis virus.

DISCUSSION

To date the predominant *in vivo* and *in vitro* evidence indicates that baculoviruses are safe for use as pesticides. However, extrapolation and usefulness of such data can be hampered by our inability to identify baculoviruses. For example, published pictures of the so-called nuclear polyhedrosis virus of *Bombyx mori* show both S (28) and M (29) NPV. It is fairly common to encounter both S and M types of NPV infecting a single species (e.g., 30, 31). The occurrence of heteropolyploids, though as yet not reported, would not be surprising in mixed infections. Where many isolates of a single morphological type of NPV from one species of insect exist, the problem is compounded. This points to the importance of developing specific and sensitive identification techniques.

Confirmation of the replication of *A. californica* NPV in *B. mori* cells in the absence of CPE and detectable polyhedrin (32) would indicate the unreliability of these frequently used indicators of infectivity. Because hemagglutination activity of NPV appears to be associated with polyhedrins (33), the use of this test as a criterion of infectivity may also be in doubt.

Qualitative differences in the neutralizing capability of antisera formed against occluded alkali-liberated and plasma membrane budded particles (34) suggest caution in interpretation of tests utilizing antioccluded-virus sera for detection of virus antigen in heterologous systems.

The evidence for possible prolonged association of some baculovirus with vertebrate cells in culture (17, 18) calls for careful and detailed studies. Effective application of newer techniques used in other branches of virology (e.g., DNA reassociation kinetics, solid phase radioimmunoassay, etc.) to study inapparent infections should be most fruitful.

Greater caution is advisable in the consideration of insect viruses belonging to groups with vertebrate or plant pathogens for use as viral pesticides. With these agents, tests must not only evaluate the direct effects and the potential for genetic recombination as suggested (35), but also the possibility of phenotypic mixing, transcapsidation, and genomic masking with known pathogens. Additionally, the occurrence of many small viruses in inapparent infections of insects (36) raises the specter of their presence as adventitious agents in preparations of other more pathogenic candidate viruses.

I believe that the EPA must take a more comprehensive position regarding biological pesticides. In particular, our registration requirements should acknowledge the many different properties of biological pesticides. With viral agents the concern is clearly infectivity and other potential effects associated with infections, as acknowledged in the Guidance for Safety Testing of Baculoviruses (4). This is not to say that toxicity should be ignored. As mentioned previously, it is the formulated product that is registered and inadvertent introduction of toxicants during manufacture of the pesticide is possible. Because viruses are exogenous sources of genetic information, which may enter cells and possibly produce effects detrimental to the organism, perhaps detailed information on the viral nucleic acid (e.g., molecular weight, configuration, density, base composition, Tm, restriction endonuclease fragments), as well as biochemical and biophysical characterization of the polyhedrin and virions, should be submitted. Although analytical methodologies for identifying the virus or its components are not required for registration, their availability is essential should a viral pesticide become suspect in health-related problems. In such circumstances techniques for detecting the virus, its structural and nonstructural proteins, and viral genome or genomic fragments would be especially invaluable.

CONCLUSION

Consideration of an insect virus for use as a pesticide should be done in a rational, prudent manner. Assessment of hazards and benefits associated with viral pesticides must be done with full cognizance of virus properties, awareness of virus-nontarget host cell associations that could occur, and the realization that viruses, like all living things, change with time.

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DISCUSSION

FIELDS: In the human volunteer studies, were any studies done on viral persistence in the gut, since many of these viruses in insects seem to attack the gut cells? You mentioned urine and blood; I did not hear stool filtrates in terms of a possible additional site that may be, biologically, a crucial one. Also, you mentioned a few positive serologic tests, but without any denominators. Could you give us some idea of the number of individuals involved. Two could be out of a thousand or out of ten, and I think the numbers would be of different significance.

HARRAP: The Ministry of Overseas Development in the United Kingdom wanted to use two baculoviruses in Africa for pest control, so it had the toxicity of the viruses tested at the Microbiological Research Establishment, Porton Down, Salisbury, United Kingdom. For this work, it used the protocol that is in the WHO Technical Report No. 531. We provided the purified virus to do the work. We also indicated to the Ministry of Overseas Development that we thought several of the testing procedures were a waste of time and that the whole protocol was deficient in one major respect. There are no tests in the toxicity testing procedures for replication of virus. With the toxicity testing that is done and the numbers that are involved, it is difficult to see how one can usefully set about this task in a reasonable period of time. To deal with this situation, all we could think of was to see if we could recover infectious virus from the test animals into a sensitive cell culture system.

We knew that the two viruses under the test, those of Spodoptera littoralis and Spodoptera exempta, would grow in one of the Spodoptera cell lines, that of Spodoptera frugiperda. So I transferred one member of my staff to the Microbiological Research Establishment on a contract for a year, and we simply used the autopsy organ material, which had been stored at -70°C for a period of 16 months. We took macerated samples both of control (nonviral-exposed animals) tissues, and tissues of virus-exposed animals, and applied both filtered and unfiltered tissue homogenates to the cells to see if we could recover infectious virus. I realize that this is a rather crude way of approaching the problem, and I am far from satisfied with it myself. However, it is better than nothing. This work has been going on for only about four and one-half months and we are only concerned at the moment with the first set of toxicity tests -- the acute oral -- which are, in fact, the larger group.

In this test, a large quantity (I think it is 40×10^9 purified polyhedra) is put by tube into the stomach of rats; thereafter, the rats are sacrificed at intervals and autopsied. Termination sacrifices are done on the remaining animals at 21 days. We then took the various organs of the alimentary tract up to the colon and triturated the tissue. To my surprise, preliminary work seems to indicate that we could recover virus from several regions of the gut. This is perhaps not surprising, but it looks as if we can recover virus into cell culture up to ten days after intubation from several organs along the alimentary tract.

The intriguing thing about this is not that the virus might have multiplied in the gut (these data don't obviously show that), but that apparently the polyhedra must have broken down in the gut lumen to release virus particles, which are infectious for the cell culture. You cannot normally infect cell cultures with inclusion body occluded virus, although I am sure there are instances in which this might occur. So, maybe virus can persist in the gut for some time in a form that is infectious for insect cells. I think I can say that at this stage. (Note added in proof: In a second batch of animals tested since this meeting took place at Myrtle Beach less convincing results were obtained.)

IGNOFFO: There have been tests on humans using orally administered viruses. If I recall correctly, Art, you did not take stool samples. In the regular production of a virus, and I am specifically referring to the people handling the Heliothis virus, there are routine clinical examinations, that include blood sampling, urine sampling, and testing for both serology based on gel immunodiffusion with its inherent problems in detection levels, and a system, which at that time, we still feel has a high level of sensitivity, that is, the use of the neonatal larvae.

There has been a study done on the fate of Heliothis virus in the alimentary tract of rats. A known high level based on LC50 units was administered to rats while they were kept on their regular diets. The food bolus passed through the animal, and the rats were sacrificed at regular intervals so that all, or at least all of the activity that we thought we put in, could be recovered. At the same time those animals were sampled for blood and various tissues and organs were sampled. So we do have an idea of the fate of a virus in the alimentary tract of a mammal, though unfortunately, not in the human. And if I recall, most of the virus, in the neighborhood of greater than 90 percent, cannot be found after three days, unless the animals are starved, in which case the passage of food is not that quick. So the virus can be retained in the animal for a longer time.

KAWANISHI: I pointed out that there was one test that took the human gastric juices and showed inactivation in vitro. I would also like to point out that during the registration of a pesticide of a virus, there are a lot more tests sent in with the application than are available in the normal literature.

Methods of Evaluating the Presence of Viruses and Virus Components

William Meinke, Ph.D.
University of Arizona, Tucson

Tissue culture, though not necessarily representative of the conditions of an intact animal body, offers a means to test the safety of insect viruses before they are released in large numbers into the environment. One advantage of tissue culture, as compared with studies involving animals, is that growth conditions of the cells and the virus can be carefully controlled and monitored. The investigator can easily assess whether viral infection leads to virally induced cytopathic effects and/or viral replication. More subtle changes, such as cell transformation or chromosomal alteration, can also be determined.

To date, infection of vertebrate cell cultures, including those of human origin, with intact baculoviruses, has neither produced viral cytopathic effects nor supported virus growth. However, in one study, isolated DNA of the nuclear polyhedrosis virus of the silk worm caused production of characteristic nuclear polyhedral bodies in cells originating from human amnion. These polyhedral bodies appeared to be identical to those found in infected silk worms and contained infectious viral particles. These findings suggest that while baculoviruses may not be infectious for vertebrates for whatever reasons, their genomes may maintain their fidelity in infected cells. Whether infecting viral DNA is free in the cell or becomes integrated into chromosomal DNA is not known.

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Because of the possibility that baculovirus genomes may retain their fidelity in vertebrate cells, studies should be implemented not merely to detect viral CPE or viral replication in infected cell cultures, but more importantly, to determine the fate of the virus in infected vertebrate cell cultures. Specifically, experiments designed to determine the fate of the viral genome in infected cell cultures should be analyzed. The finding that viral genes are rapidly degraded in infected cell cultures would definitely support the notion that baculoviruses pose no serious problem to vertebrates, including man and domestic animals. However, should viral genes persist in infected cells, the possibility that certain insect viruses may be potentially hazardous could not be discounted.

Fortunately, the methodology for evaluating the fate of viral genetic information in infected cell cultures is relatively straightforward. The presence of viral DNA in infected cell cultures can be detected by extremely sensitive DNA-DNA hybridization techniques. The sensitivity of DNA-DNA hybridization is such that as little as one viral genome equivalent per 5-10 cells can be detected. For these types of studies, two parameters must be met. First, viral DNA labeled to very high radiospecific activities (greater than 10^7 counts per minute per μg DNA) is required to serve as a "probe" for detection of unlabeled viral DNA sequences in cellular DNA. Secondly, large amounts of total cellular DNA are required in order to detect the relatively small viral genome within DNA extracted from the tremendously larger cellular genome.

For the remainder of the discussion, I would like to present some of the methods we have used to detect viral DNA sequences in infected cells. While these studies were not done with baculoviruses, the methods and results that I will present could easily be applied to a study of the fate of insect viral DNA in infected cell cultures.

For DNA-DNA reassociation studies, viral DNA with specific activities greater than 10^7 cpm/μg is required. *In vivo* replication of DNA viruses in the presence of radioactively labeled DNA precursors generally results in progeny viral DNA molecules with specific activities less than 10^6 cpm/μg. Accordingly, we have developed methods for labeling viral DNA *in vitro* by employing "nick-repair" enzymatic reactions (see Table 1). Viral DNA is first treated with pancreatic deoxyribonuclease I at ratios of 1:500 to 1:1000 in order to introduce a limited number of single-stranded scissions. The nuclease is then inactivated by heating to 65° for 10 min. Viral DNA is subsequently labeled by initiating repair synthesis by addition of

TABLE 1. *IN VITRO* LABELING OF VIRAL DNA

Purified viral DNA in 0.05 M tris-HCl, pH 7.5, 0.05 M KCl, 0.005 M $MgCl_2$

↓

Add pancreatic deoxyribonuclease in 0.05 M tris-HCl, pH 7.5, 0.005 M $MgCl_2$ at a ratio of 1:500 (wt/wt) (DNA to DNase). Incubate at 37°C for 15-30 min to introduce "NICKS."

↓

Heat to 65°C for 19 min.

↓

Dialyze "nicked" DNA against 0.07 M potassium phosphate, pH 7.4, 0.007 M $MgCl_2$

↓

Make DNA 0.1 μmol 2-mercaptoethanol and 0.005 μmol with each necessary unlabeled deoxynucleotide triphosphate. Add one or two labeled deoxynucleotide triphosphate(s).

↓

Begin "repair" by addition of 20 units DNA polymerase and incubate at 15-17°C for 60 min.

↓

Reaction stopped by addition of Sarkosyl to 1%.

↓

Pass reaction mixture through Sephadex G-50 equilibrated with 0.02 M tris-HCl, pH 8.0, 0.001 M EDTA, 0.1% Sarkosyl.

↓

Pool labeled DNA peak.

↓

Extract with phenol.

↓

Precipitate with 2 volumes 95% ethanol.

Escherichia coli DNA polymerase I and the necessary deoxyribonucleotide triphosphates. Reactions are at 15-17° in order to reduce the possibility of displacement synthesis. Repair synthesis is terminated by the addition of Sarkosyl and the DNA subsequently separated from the reaction ingredients on Sephadex columns. Depending on the type of labeled deoxyribonucleotide triphosphate employed, specific activities of DNA labeled by these procedures range from about 2-80 x 10^6 cpm/μg DNA. For example, DNA labeled with ^{3}H-TTP generally has specific activities of about 2-6 x 10^6 cpm/μg while utilizing ^{32}P-TTP or ^{125}I-dCTP as the labeled precursor yields DNA with specific activities of 20-80 x 10^6 cpm/μg.

Our in vitro labeled viral DNA probes generally sediment at about 5.6 S in alkaline sucrose gradients (Figure 1). This sedimentation velocity value corresponds to an average single-stranded piece size of about 430 nucleotides, which also is about the size of an average gene sequence. We have shown that in vitro labeled viral DNA denatures with the same sharp profile as observed with viral DNA labeled in vivo, and furthermore, denatured in vitro labeled viral DNA will reassociate to greater than 90% with normal second order kinetics.

Thus, in vitro labeled viral DNA should serve as an effective "probe" to detect unlabeled virus-specific DNA in total cellular DNA derived from infected cultures. Total cellular DNA can be extracted by a urea-phosphate-hydroxyapatite column procedure. In brief, cells are suspended in 8 M urea, 0.24 M phosphate, pH 6.8, 10^{-3} M EDTA, 1% sodium dodecyl sulfate, 2% isoamyl alcohol and the cells lysed in a Waring blender (60 seconds, maximum speed). The solution is applied to a column of hydroxyapatite equilibrated with the same solution (minus EDTA and isoamyl alcohol). Cellular DNA binds to the column while cellular proteins and RNA are eluted with several washes. Urea is then removed by washing the column with 0.14 M phosphate, pH 6.8, buffer. DNA can then be eluted with 0.48 M phosphate, pH 6.8, buffer (Figure 2). DNA can be further purified by phenol extraction and then reapplied to a second hydroxyapatite column equilibrated with 0.14 M phosphate, pH 6.8, buffer. Following a second elution from the column, the DNA appears free from protein and RNA contamination. Using these procedures, we usually obtain about 8 x 10^{-6} μg DNA per mammalian cell (i.e., total cellular DNA).

In order to detect small amounts of viral DNA in cellular DNA, nanogram quantities of denatured labeled viral DNA usually are reassociated in the presence of milligram quantities of cellular DNA. There are several conditions that will affect the reassociation of the labeled viral probe to form stable duplex molecules. These include the concentration of DNA,

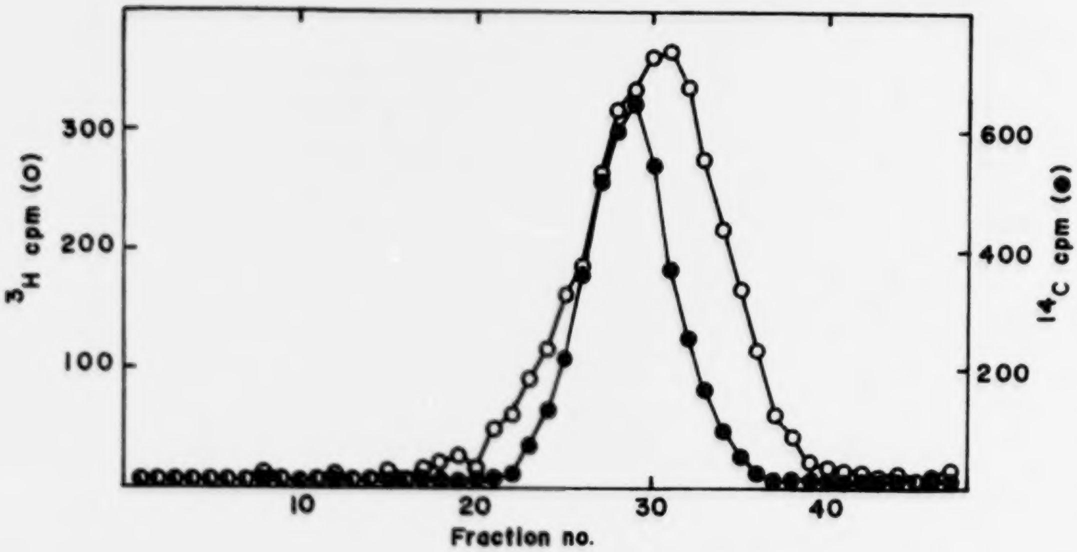

Figure 1. Sedimentation of *in vitro* labeled viral DNA in alkaline sucrose gradients. Sedimentation is from right to left in 5 to 20% linear alkaline sucrose (0.3 *N* NaOH, 1.0 *M* NaCl, 0.001 *M* EDTA, 0.015% Sarkosyl) gradients in an SW56 rotor at 15°C for 6 hr at 49,000 rpm. ●—●, ^{14}C-labeled marker DNA 570 nucleotide in average length; o—o, *in vitro* ^3H-labeled viral DNA.

duration of incubation, salt concentration, temperature, size of the DNA fragments, solvent viscosity, the guanine + cytosine content of the DNA, and the nucleotide sequence complexity.

The kinetics of DNA-DNA reassociation follows the equation:

$$Co/C = 1 + KCot$$

where *Co* is the initial concentration of single-stranded viral DNA probe, *C* is the concentration of the single-stranded viral probe at time *t*, and *K* is the reassociation constant. A plot of *Co/C* versus *t* will result in a straight line if the DNA-DNA reassociation reactions are following second order kinetics. Should unlabeled viral DNA sequences be present in DNA extracted from infected cells, the reassociation rate of the probe will be increased in direct proportion to the amount of unlabeled viral DNA present.

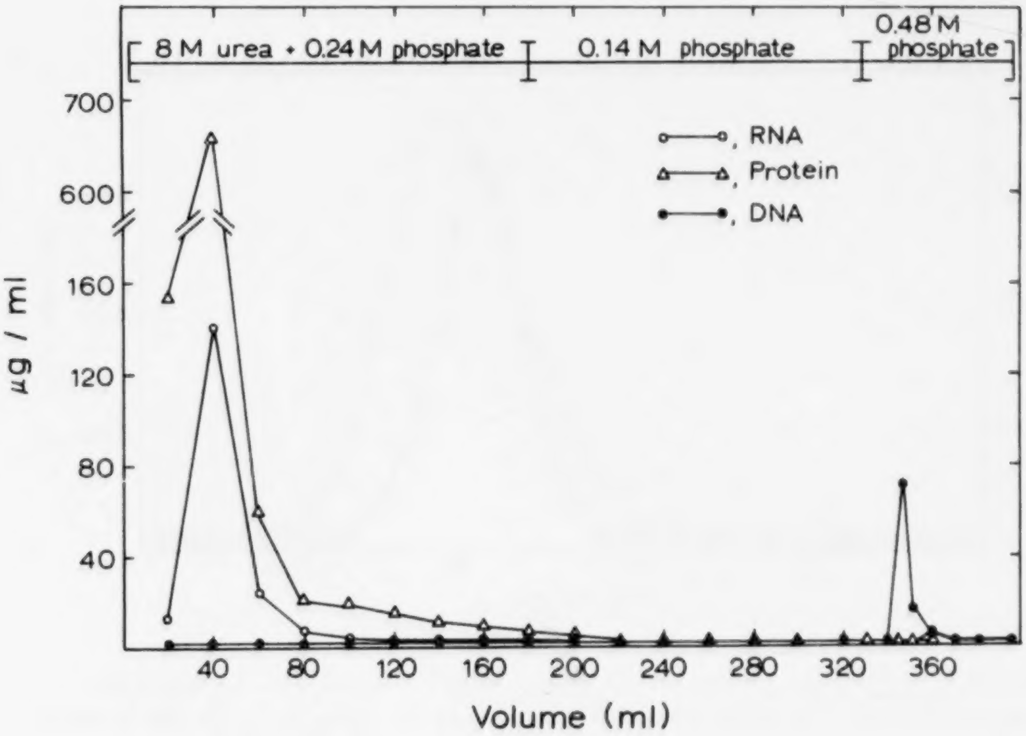

Figure 2. Hydroxyapatite-urea-phosphate column chromatography isolation of cellular DNA: chemical analysis of fractions. Fractions were chemically assayed for RNA ($FeCl_3$-orcinol method), DNA (diphenylamine reaction), and protein (Zak and Cohen modification of the Folin phenol-reagent method).

Thus, from the increased reassociation rates, the total amount of viral DNA present can be calculated.

The actual methods to follow reassociation of labeled probes are really straightforward. Labeled viral probes are denatured in the presence of unlabeled cellular DNA and allowed to reassociate. At various time intervals, samples are removed and applied to columns of hydroxyapatite. Single-stranded DNA is eluted with 0.14 M phosphate, pH 6.8, buffer while reassociated duplex molecules are subsequently eluted with 0.48 M phosphate, pH 6.8, buffer. If the cellular DNA contains viral specific DNA sequences, the rate of reassociation will be increased over that of control reactions due to the increase in the concentration of viral DNA sequences.

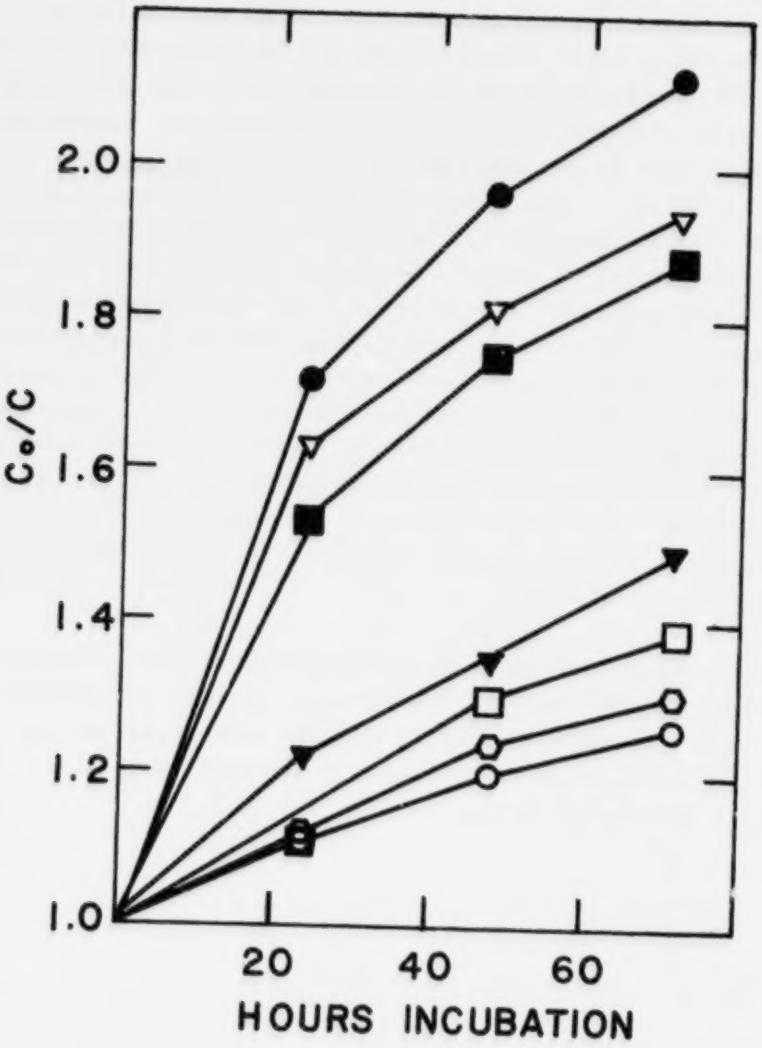

Figure 3. Reassociation of ^{3}H-labeled DNA from the human papilloma virus (HPV) in the presence of unlabeled DNA from HPV infected Wi38 cells. The results are plotted as the ratio (Co/C) of the total ^{3}H-labeled HPV DNA concentration (Co) to the concentration of single-stranded ^{3}H-labeled HPV DNA (c) at various incubation times. Cellular DNA extracted at various time intervals after HPV infection: ●—●, 90 min post-infection; Δ—Δ, 24 hr post-infection; ■—■, 48 hr post-infection; ▲—▲, 96 hr post-infection; □—□, 168 hr post-infection; ⬡—⬡, 336 hr post-infection; o—o, control reaction consisting of HPV ^{3}H-labeled DNA reassociating in the presence of Wi38 cellular DNA extracted from uninfected cells.

Data derived from a typical DNA-DNA reassociation experiment is presented in Figure 3. In this case, human cells (WI38) were infected with the human papilloma virus (wart virus). Like the baculoviruses, the human papilloma virus (HPV) produced no CPE in infected cells and apparently does not replicate in cell cultures. The bottom curve shows the reassociation rate of labeled viral DNA in the presence of Bacillus subtilis DNA (i.e., control reaction). The top curve shows the reassociation rate generated when viral DNA was reassociated in the presence of cellular DNA extracted immediately after the infection period. From the increased rate of reassociation, it was calculated that there were at least 27 genome equivalents per cell 90 minutes after infection. Twenty-four and 48 hours after infection, there remained 22 and 20 genome equivalents, respectively, per cell. In fact, viral genetic information could be detected even two weeks after infection. Thus, while there was no increase in the amount of viral genetic information in the infected cells, the ability of the viral genes to persist suggests that this virus could remain in a quiescent or latent state and that some extraordinary event could induce it to either replicate or, alternatively, to transform cells.

In summary, it may be advisable to utilize DNA-DNA reassociation experiments to determine the fate of insect viral genetic information in infected vertebrate cell cultures. These studies could rule out the possibility that certain insect viruses may possess hazardous potentials to either man or other beneficial living forms.

DISCUSSION

KAWANISHI: Have you tried any of the baculovirus experiments yet?

MEINKE: We are now at the point of in vitro labeling of the baculovirus DNA. Oddly enough, you'd think that what worked best before would work for baculovirus DNAs, that a microgram of one should behave like a microgram of another. They do not, so now we are trying to get a good probe.

IGNOFFO: Can you tell us what your model systems are?

MEINKE: With relation to what?

IGNOFFO: Baculovirus. Which baculovirus and cells are you using?

MEINKE: We are going to use the *A. californica* baculovirus, and we are going to use WI38 cells as a representative human cell line.

IGNOFFO: Are you going to use an insect standard cell line also?

MEINKE: Yes, we will use TN368.

Hazard Evaluation for Viral Pesticides: Test Data Requirements

Reto Engler, Ph.D., and Martin H. Rogoff, Ph.D.
Environmental Protection Agency
Washington, D.C.

Over the last decade, interest in exploiting the pathogenic properties of insect viruses as pesticides has raised two related issues: one is the human safety aspect of using insect viruses as pesticides, and the second is environmental effects on nontarget species. To deal with these issues one must resolve the problem of which test data requirements would have to be satisfied in order to register viral products and to promulgate tolerance regulations for those viruses with a potential for leaving residues in food and animal feed. The EPA separates these two issues, related to the hazard assessment responsibilities of the EPA, in order to maintain a proper perspective and to allow us to assign priorities to the various activities related to the use of viral pesticides. It is our task at this meeting to address mainly the second area of concern, the data requirements necessary for registration and tolerance regulation promulgation purposes.

In order to discuss data requirements from a proper perspective, we must first clarify EPA's statutory and regulatory mandates as they relate to registration and tolerance activities. First, a pesticide can be registered if the EPA can establish a finding (upon examination of supportive data) that, when used in accordance with its label directions and good agricultural practices, use of the pesticide is not likely to result in unreasonable adverse effects on man or on the environment. This concept does not imply that the EPA guarantees the absolute safety of the pesticide.

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However, it does imply that the registration of a pesticide allows its use under conditions representing a risk to the public which is acceptable to the administrator. The statute further implies that even pesticides that pose an unacceptably high risk under general conditions of use may be registered if they are classified as restricted, which means that they can only be applied by trained individuals specifically certified as pesticide applicators.

Similarly, the practice of promulgating tolerance regulations for an allowable residue or exempting specific pesticides from tolerance regulations does not imply absolute safety. Establishment of a tolerance implies that there is a practical certainty that residues of the pesticide below the tolerance level will not produce adverse effects on the public's health. Again, the implication is that of an acceptable risk. The concept of incremental risk is also involved in an examination of a pesticide proposed for registration. Thus, when reviewing a pesticide's supportive data and its chemical, physical, and biological nature, data reviewers must include evidence that there is a practical certainty that the pesticide will not produce deleterious effects on the public's health as a result of its use or due to residues ingested in tolerance situations.

The statute, however, takes one further step, and this step is reinforced and formalized in the regulations. The statute holds that in those instances where risk is demonstrated as unacceptable, such pesticides may still be registered and tolerances promulgated if benefits from its use are found to outweigh the risks involved. Such a finding is reached via the rebuttable presumption procedure.

The preceding indicates that the Agency recognizes a clear distinction between the universe of data required to support a registration or a tolerance and the universe of data that is required to answer the question, "Is this pesticide safe?" With this background information, we can proceed to current data requirements for viruses proposed for registration.

Since the early seventies, several attempts have been made to delineate data requirements necessary to assess the potential hazard posed by the use of viral pesticides. The publications (1-3) on this subject have the following two things in common: they are vague, and they address two main areas of concern -- the need for precise virus identification methodology, and the need for an adequate demonstration of lack of infectivity for nontarget species including man.

The following discussion addresses current minimal data requirements considered adequate for hazard evaluation of viral pesticides. As previously stated, one should not attempt to relate the utilitarian value of these test data directly to the value of research studies. Research studies are designed to answer questions related to determination of the absolute safety of use of baculoviruses as pesticides. The hazard evaluation consideration is a dynamic one, and the requirements described will always be subject to change as the scientific knowledge base expands and alternate and more relevant approaches are brought forward for consideration.

The first requirement in any application for registration is the identification of the pesticidal active ingredient (4). It has been stated that the identification procedures and taxonomic tools applicable to insect viruses need improvement. At present, we cannot state definitely whether biological, biochemical, or serological methods will be the ultimate identification tool for regulatory purposes. At this time, the requirement for identification can be satisfied if the virus is identified by a combination of valid available methodologies used for virus identification. The use of a combination of biochemical and biological methods appears to be most promising (5-7).

In addition to the qualitative identification of the virus, a quantitative description of the virus is required because use rates for the virus must be established and because of certain regulatory labeling requirements. In short, an analytical method (in all instances to date, a bioassay) must be developed. The bioassay is not in actuality an additional data requirement, but it is an essential tool required for several regulatory considerations as well as for commercial development of the pesticide. These considerations include quality control and a determination of the stability of the product, the residual virus levels on food and/or animal feed, and the fate of the pesticide in the environment (8).

The second requirement in submitting a pesticide for registration is a proposed label for the product. Although the label addresses many other aspects of pesticide regulation, such as use directions, rates of application, and pests controlled, we will only consider those portions of the label related to hazards and precautions. A complete statement of the ingredients in the formulation, the active ingredient(s), inerts, and contaminants must be submitted in order to ascertain whether the inert ingredients used in the formulation are approved for the intended use. The active ingredient(s) are normally listed by weight percent. This poses a problem for biological pesticides. In the case of biologicals, the bioassay is used as a bridging tool to relate biological activity to actual weight of the active ingredient. As in the case of *Bacillus thuringiensis*, the active ingredient must be listed in both ways, as weight percent and in terms of biological activity. *Bacillus thuringiensis* has an International Unit convention. With *B. thuringiensis* weight is also under a convention.

The label must also contain the appropriate signal words and precautionary statements derived from the results of the tests which are described below.

The third submission requirement is related to hazard evaluation. The testing can be divided into two parts. One deals with the hazard evaluation on the formulation, the other with the hazard evaluation related to tests performed on the active ingredient of the pesticide. The hazard evaluation for the formulation is relatively straightforward. The EPA requires an acute oral exposure study, an acute dermal exposure study, and primary skin and eye irritation studies on the formulation. These constitute the full battery of tests mandated for all products registered. Additional tests are considered conditional. The acute studies are used to determine the appropriate label precautionary statements for the product. If the formulation in use can produce a respirable dust, mist, or vapor, an acute inhalation study is also required. If repeated dermal or inhalation exposure is likely, subacute dermal and inhalation tests and dermal sensitization tests are required. In short, a viral pesticide as a formulated product has to undergo the same type of testing as would any other pesticid We emphasize that these tests do not deal with chronic exposure implications of actives or inerts but provide the general basis for labeling to protect users from acute and subacute effects of the formulated product under expected conditions of handling and use.

The second part of the hazard evaluation testing is carried out on the virus itself (the active ingredient "technical chemical") and is basically for chronic effects. The extent of this testing depends on several factors, including the use pattern proposed for the pesticide and expected environmental effects, if any, upon application of the virus. This testing also includes determination of natural levels of virus in the environment and inactivation of the virus in the environment. Last, but of major importance, evidence must be provided to demonstrate that the virus is innocuous for nontarget species. We feel that the potential hazard resulting from oral exposure to a viral pesticide can best be demonstrated by an appropriate modified, acute feeding study format. The study should follow a protocol where relatively high doses of biologically active material are administered to experimental animals. The protocol must include an investigation of the stability of the virus in the test animal, rate of inactivation, and the transfer potential of the virus through the animal or into the tissues of the animal. In short, even lacking gross infection, invasive and pervasive properties of the virus should be determined. The study must also determine whether the test animal experiences any symptoms related to clinical or subclinical infectious diseases in mammals. Classic toxification symptoms are not expected. Infectious symptoms would include an increase in normal body temperature and an increase in immunological responses. The selection of the test species is important because of the well-known refractory properties of many viral hosts and viral spectrum. Thus, one of the species selected should be a primate.

In addition, subacute feeding studies should be performed on at least two species of non-primates using relatively high daily exposures. These latter studies may not always be necessary, for example, in those cases where prolonged oral exposure is unlikely, or when other studies indicate that exposure after viral application is equal to or less than exposure of the population at risk under natural conditions.

The major hazard of concern with viral agents is infection. Thus, if infectivity testing provides no overt evidence that the pesticidal virus interacts with representative vertebrate hosts, the usual studies assessing chronic effects such as oncogenicity, teratology, and other long-term effects are not considered as prerequisite test requirements for registration.

Fish and wildlife studies are required depending on the use patterns of the virus pesticide. These studies include acute and subacute studies in representative avian species, as well as representative vertebrate and invertebrate aquatic species. The studies must assess acute hazards from the formulation and the possibility of an infectious process in non-mammalian species, as opposed to the standard assessment of the virus as a toxicant.

The fourth part of a submission for registration must define biologically active viral residues in a quantitative manner. The purpose of these studies is not to establish numerical tolerances for residues, as is the purpose with chemical pesticides. These studies are intended to provide an understanding of the fate of the virus in the environment. They should be designed to reveal what they can about inactivation processes and to quantitate viral fate if possible. Submission of evidence that the purposeful application of virus reduces the total amount of residual virus on food and animal feed items in the environment is highly significant in hazard assessment in that the practical certainty that no unreasonable adverse effects can thus be expected would be considerably strengthened. Environmental residue studies also serve to fulfill the requirements for environmental chemistry data, even though the nature of the tests will differ considerably due to the biological nature of the active ingredient. However, the basic questions concerning residue quantitation, dissipation rates, and mobility would apply to a virus as they do to a standard chemical.

The EPA will also consider any other evidence which would contribute to correctly assessing the hazard posed by viral pesticides. Acceptable supportive evidence would include recent published or unpublished studies of the basic virology of the pesticidal virus, or related viruses, or other investigations relating to the particular virus pesticide. Studies of interest include attempts to inoculate vertebrate and invertebrate tissue culture cells using sensitive methods to detect virus-cell interactions, or studies using unusual routes of administration, such as i.v., i.p., or even intracerebral inoculations. Basic biochemical studies on viral DNA, including such identity criteria as annealing or hybridization, may also be of use in hazard assessment. Although these studies are helpful and are taken into consideration, they are not considered mandatory regulatory tests.

In accordance with recent advances in molecular biology methodologies, it is conceivable that insect virus DNA might be hybridized with mammalian or other DNAs. In a hazard assessment mode, this finding of in vitro hybridization would not be weighted heavily in terms of risk. On the other hand, if hybridization with presently available techniques is not possible, such a finding would be useful in the evaluation.

This is a statement of current general approach. It is neither final nor static. Hazard evaluation under its present concept is a new art and will be in a highly dynamic state for the foreseeable future. Therefore, specific test requirements or test techniques will change as progress is made and more fundamental understanding of the properties of insect viruses emerges. Newer techniques and tests must be properly assessed as to their utilitarian value in the regulatory mode. New techniques will require standardization with respect to protocol before they can properly become standard requirements of data to be submitted by a registrant.

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DISCUSSION

FIELDS: I have one practical question about primate testing. Do you anticipate any difficulty in obtaining sufficient numbers of primates for testing, considering the difficulties in obtaining such animals?

ENGLER: I don't know the answer to this question, but considering the few viruses which might be registered, I do not foresee that a vast number of primates will be needed.

FIELDS: I know, for example, in the vaccine evaluation studies of polio virus vaccines, and in experimental studies where primates are required, there really is a current crisis in the availability of such creatures, and I think it is going to become (since you are talking about subhuman primates as one of the key experimental hosts that most closely resemble humans) a very large problem.

RAPP: You are going to have to decide whether you are going to go down the evolutionary ladder from the chimpanzee, to the baboon, to the rhesus monkey or the African green monkey, to the marmoset, or wherever you are heading. As you move further up toward the chimpanzee, things get much more expensive; and this is in line with your comments that some of the suggestions have not been specific, and sooner or later someone has to take this by the tail and decide where and on which animals some of these tests will be performed. Unfortunately, there probably isn't enough known about the biology of these agents, especially in noninsect species, to provide very good clues as to which subhuman primates to use or, for that matter, which rodent. Why use the rat, rather than the mouse or the hamster? Which animal would be the best choice (it might not be the same animal for a larger number of these different baculoviruses)? We might discuss this further later on, but this is a very difficult problem. I agree with Dr. Fields. Many of these animals are on the endangered species list and, for all practical purposes, are impossible to get. Only a few are being successfully bred in this country in limited numbers, like the baboon or the marmoset, and they are in short supply. If you begin to get into this fairly extensively, of course, the next question is whether to use newborns, young adults, or older adults -- which adds to the problem of cost. You asked a simple question, but I hope you realize the complexity of it. I don't know at the moment how one could advise which animal to use for this test.

JOKLIK: I think you gave us a very clear exposition on what the current regulations are concerning viruses as pesticides as a whole. I don't want you to get into any scientific discussions but what interests me is what the mechanism is within the EPA for changing these regulations, for upgrading them, and so on. Is this done solely within the registration branch of the EPA, or are outside experts brought in? Is there a mechanism for continuous upgrading review, evaluation of what is already being done, and so on? How is this managed administratively?

ENGLER: There is a way to continuously upgrade requirements. We are now in the process of another reorganization. Subsequently, we will have a hazard evaluation division and, hopefully, existing in that division will be a means of seeking outside advice in these areas, not only for viral pesticides, but for chemical pesticides as well. So I can assure you that the continuous update of knowledge and research findings will be incorporated into our evaluation.

SUMMERS: Since the early 70's, I know that you have been actively involved in writing numerous drafts of guidance testing for biological agents; so I am more familiar with your historical evolution with this problem than a lot of people here. It's my understanding that you have reached the sixth draft, which was published in Baculoviruses for Insect Pest Control: Safety Considerations, as the guidance for safety testing. And yet, in your safety testing protocols, I haven't seen any attempt to implement some of the basics of those recommendations relative to infectivity for viruses. So if you have a constant mechanism for update, why haven't we seen some reflection of this in the required safety procedures, at least as they're written into law?

ENGLER: There is nothing written into law.

SUMMERS: Well, whatever, the Federal Register.

ENGLER: There is nothing in the Federal Register.

IGNOFFO: Could you make a distinction between guidelines and regulations.

ENGLER: In fact, there are no guidelines for safety testing of viruses, no legally binding guidelines. What is printed in Baculoviruses for Insect Control is called a "guidance" -- that was done on purpose.

COLLINS: At least two agents have been registered thus far for use as viral pesticides. What I would be interested in, and I think might be a lot of help, would be a review of the testing of these two agents. How the agents conformed to guidelines at the time they were registered could be examined to indicate how carefully these guidelines are either being followed or ignored before registering agents for this kind of use. I think it would be very useful if we could know what kind of data went into the decision at the time that these agents were registered.

ENGLER: I would be happy to do that, but I do not think this is an opportune time.

COLLINS: I just brought it up now so that you would be aware of its usefulness.

ENGLER: May I mention two related problems? First of all, the problem which Dr. Rapp touched on: Is anybody in this room or anywhere else prepared to say, whether a chimpanzee is a better subject than a marmoset, and, if so, how much better? These are the questions that we are wrestling with.

The second item is that at the time Heliothis zea NPV, for example, was registered, we did not know many things we know today about these viruses. However, a decision was made on the currently available information that the risk was acceptable. The aspect of changing knowledge has to be considered. We are still in a developing phase for viral pesticides, much more so than with chemicals, yet even with chemicals we constantly change some of the requirements.

COLLINS: I believe the third virus is currently close to registration. It might also be useful to this discussion if you could tell us what sort of data are available on that. This is at a time when these registrations are under reconsideration.

The other point refers to something you said. If I understand you correctly, the current guidance situation is that if no signs of infectivity have been found in nontarget species, and the feeling is that these should not be looked at for oncogenicity. It seems from what we've heard, in terms of the biology and some of the biochemical features of these viruses, that there is that much justification for such an approach. It should be of particular interest to look at the possible oncogenicity of these agents in nontarget species when you don't find signs of active infection. I think that a particular guideline, if I heard you correctly, might be worth reconsidering.

IGNOFFO: I would like to make a point about the first questions he brought up and put it in historical perspective as well. When the attempts were made to initially register a viral pesticide, EPA did not exist. Registration was initially done with FDA. Protocols did not exist, so the type of reasoning that you want to apply has to be placed in the perspective of these protocols being developed. With a change of reorganization, the kinds of tests performed were done in consultation with FDA microbiologists and virologists, and NCI microbiologists and virologists. The ultimate decision had to be made, of course, on the basis of data that were there.

COLLINS: Has there been any reconsideration or reevaluating of these agents relative to new guidelines?

IGNOFFO: There has been consideration of reevaluating and redoing critical tests that were done previously, when and if the sensitive techniques become available. That has always been a consideration. It has been recommended from the start that as techniques become more sensitive and more specific, they should be employed in a monitoring system and also in critical animal tests; nontarget animal tests should be redone with those new, sensitive systems.

COLLINS: Should this be done concurrently with the use of these agents, or should their use cease until these tests are done?

IGNOFFO: You bring up the point that Dr. Fields brought up yesterday. How certain can you be? We cannot have absolute certainty. So at some point, we are going to have to make a decision. And that decision can only be based on the technology that we have and the information we have available at that time. We may make a mistake. What certainty do you have that the vaccines that have been pumped into your children will not develop into oncogenic viruses in 20 years? Or what reassurance do you have that they will not develop into any monster?

JOKLIK: This is not really relevant.

IGNOFFO: I believe it is relevant to the point he was trying to make.

JOKLIK: I think we should perhaps discuss that further this afternoon. Ralph, did you have a comment?

SMITH: It is also something that should invite a more comprehensive discussion. As we heard just now, part of the licensure and registration is an evaluation of risk. What I think is clear is that we are here to provide guidance as opposed to guidelines. We might assist both the investigators and the administrators in some discussion, and perhaps, even a more concrete formulation of what is an acceptable risk and what is an unacceptable risk. In other words, one of the things that would assist the administrators, and even perhaps workers in the field, is that if one incident occurs, this insecticide should not be used. If that happens, it could be used -- where we can perhaps begin to define that if certain things happen there should be something done.

ENGLER: I think that's a very sensible approach.

HARRAP: Can you clarify a point of information for me? Is it correct that the only mandatory tests are those required on the formulation, and the tests required on the active ingredient are conditional and not mandatory?

ENGLER: Yes, in a sense that is correct, the conditional tests are contingent on the proposed use. If you use your formulation in a little black box, there are no other than acute tests required. In other words, there is always a condition for some of the so-called conditional tests.

KAWANISHI: Isn't it correct that the proposed guidelines, published in 1975, specifically mention formulated product and technical product, at least for the chemical pesticides?

ENGLER: That is true. The formulated as well as the active ingredient has to undergo certain tests. However, the acute tests are and especially will be only required on the formulation because we need these data for the purposes of labeling. If the purpose of testing is directed to the overall safety of the active ingredient, we will have to test the active ingredient. So again, tests on the active ingredient are conditional, but any time this pesticide is used outside of a vacuum, some of these conditions will be invoked. Not all conditional tests will apply to each use; therefore, we have the apparent ambiguity of conditionality.

PART VI
PANEL DISCUSSION

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Safety Procedures and Future Recommendations

Moderator: W. K. Joklik, Ph.D.

ENGLER: We consider safety tests a pragmatic approach to the hazard evaluation; furthermore, that the actual tests will not be the only safety considerations. The safety picture of viral pesticides and any other pesticide is a conglomerate of tests and of considerations, such as use rates, dissipation rates, use location, etc. The actual safety tests are only a part of the total considerations that are used for making a final recommendation for registration. After this short preface, I will discuss the pragmatic approach, which we are presently using.

First, there are four basic acute exposure studies with the formulations. Then we have as many as four conditional acute and subacute tests on the formulation. These include inhalation, subacute dermal, sensitization tests, and the like. Third, we consider feeding tests on the active ingredient which may be acute and subacute. Depending on the expected exposure, there may also be inhalation tests on the active ingredient at the acute and subacute levels. What should be emphasized is that especially in the third category of tests, we are concerned with the invasion and persistence of the virus in the test animal. In the acute tests on the formulation, obviously that is not the most important purpose, but in the tests on the active ingredient, we are looking for the invasion, the persistence, and the degradation of the virus as much as technique allows at present.

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JOKLIK: As I understand it, are one and two, in essence, toxicity tests?

ENGLER: That is correct.

JOKLIK: In other words, you feed or administer relatively large amounts of your formulation to your test animals. I presume these are animals rather than cultured cells.

ENGLER: These are animals.

JOKLIK: And you observe them over a relatively short period of time for any adverse effects. Is that correct?

ENGLER: Yes.

JOKLIK: What would the period of time be? Would it be up to a couple of weeks or a month?

ENGLER: About a couple of weeks. Some of the conditional tests, which may be subacute dermal exposure tests on the formulation, may continue for as long as three weeks, with another week for observation. A sensitization test may be required as well.

JOKLIK: Are serum enzyme elevation tests included, or is that something different?

ENGLER: You mean antibody?

JOKLIK: No, alkaline phosphatase.

ENGLER: No, that would be included under tests carried out in category three.

JOKLIK: How does three differ from one and two?

ENGLER: For tests under categories one and two, the material is formulated, and may contain as little as 0.5 percent of the active ingredient. In the category three tests, the active ingredient or technical material is administered to the test animals.

JOKLIK: So in our particular case, it would be highly purified virus?

ENGLER: It would be what is being proposed to be used in the formulation. It may be a highly purified virus, it may be virus plus insect fragments, partially purified insect virus less the fragments, and so on.

(Inaudible.)

ENGLER: Well, maybe the separation into categories is in a sense artificial; however, the reason I am separating categories one and two is to stress the point that these tests simply assess the toxicity of the formulation. As Dr. McCarthy said yesterday that sometimes all kinds of impurities are put into the formulations, and we need to know what the toxicity of the total complex of the formulation is -- the acute toxicity of that complex. If you have a formulation that has one percent of virus in it, it is somewhat irrelevant to look for what happens to the active ingredient. One may not find the needle in the haystack. This is why I am separating the two approaches, that is, testing on formulation and testing on the active ingredient.

HARRAP: I have in front of me WHO Technical Report No. 531. This is one of the documents that contains a version of the test protocol. In the acute oral toxicity test, there used to be quite elaborate procedures for, for example, organ histopathological examination, a termination report on hematology data, clinical chemistry, gross necropsy findings, microscopic examinations, etc. Is all this still done, or is this detail no longer relevant?

ENGLER: The test you are talking about is identified in category three on the blackboard, and it is done with the active ingredient.

HARRAP: That is the active ingredient one. It would not be done for the formulation then?

ENGLER: No. Again, why do it with the formulation in which less than one percent of the virus may be present?

HARRAP: When you compare the two types of feeding tests, the formulation and the active ingredient (the virus), you said that the virus need not necessarily be purified. Could the difference between the formulation of the virus and the active ingredient simply be the presence of the ultraviolet screening compounds or stickers, and so forth? Or is it a different preparation of viruses taken from the insect?

ENGLER: The difference between the formulation and the technical material is that spreaders, stickers, screens, and diluents have been added to make a formulation, but the technical material of the active ingredient is 100 percent of the technical virus as it is produced.

HARRAP: So, is the requirement that we used to have for that active ingredient being in some purified form now totally dropped because acetone precipitated slurries of polyhedra were specified originally?

ENGLER: That depends on the particular virus because some virus preparations in the past -- the very few that we have registered -- have been more or less purified. For example, the gypsy moth at one time had been highly purified, then they decided to purify it to a lesser degree. In other words, the tests should be done with that type of material that is eventually used as a component of the formulation.

HARRAP: I can see that when you test the formulation, but I am rather surprised that you do not do the test on the active ingredient in a more concentrated or purified virus sample. Any possibility of difficulty with your formulation would come out in your formulation testing. If you were going to look for something that is intrinsic to the active ingredient, it would seem better to just have purer active ingredients to deal with.

ENGLER: It appears that it all boils down to the definition of the active ingredient and the technical material.

IGNOFFO: We are looking for possible replication and/or development in a non-homologous host and ways to increase that probability. Dr. Engler is concerned with registration of products that come off the production line. From the standpoint of ensuring safety, it is better that the product not be purified since purification could eliminate extraneous, harmful biotypes.

HARRAP: You just made the point that one of the things we are looking for in the active ingredient test is some pathological manifestation, and it would seem to me that you are more likely to get a pathological manifestation caused by the virus from using the virus alone. That is the only point I am making. I just cannot see the logic of your distinction between the formulation and the active ingredient in terms of how you prepare the virus.

COLLINS: Why do these two approaches have to be mutually exclusive? Why not have three approaches? That seems to be one easy way out, particularly concerning the animals used in these tests. Another easy way out would be to use the active ingredient in its most purified form, then the ingredient as it goes into the formulation, and thirdly, the formulation. That would at least give you a distinction between any contaminants in the formulation that may have toxicity effects, the active ingredient at a concentration that is somewhat more realistic relative to the formulation, and the intrinsic properties of the active ingredient in its purest form.

SHOPE: Regarding tests on the active ingredient, is an immunosuppressed host or otherwise compromised host used in these tests, in addition to intact animals with intact immune systems?

ENGLER: We have considered using immunodepressed hosts. However, there has been no final decision on the true relevancy of such a requirement for all viruses.

IGNOFFO: If you consider very young mice as immunodepressed hosts, then there have been tests with viruses. The only other test that I know of with a biological agent was with a species of nematode which attacks mosquitoes. This nematode was tested against drug-immunodepressed rats.

SMITH: Who does this testing?

ENGLER: The testing is required from the person or agency that is requesting registration. This may be private industry, it may be another government agency, for example, the Forest Service for the tussock moth and gypsy moth. The actual testing, however, is mostly contracted out by the registrant.

SMITH: Let's say another government agency, which may or may not have the funds to do the testing, comes to EPA and says, "We would like to use this as a biological control material." You would not do the testing for them? They would have to come to you with the data, and if that is the case, who evaluates their data?

ENGLER: It is true, they have to come in with the data, and we, the EPA, would then evaluate the data.

JOKLIK: I do understand the difference here. This relates to the testing of an actual formulation, whereas some of the questions were directed to the properties of the active ingredient of the virus actually in these formulations, which may require additional testing. To summarize, what total information concerning a formulation is required or has to be on hand before it is licensed? You said this is the sum total of information that is required.

ENGLER: I have not addressed any auxiliary testing or evidence like environmental persistence studies or fish and wildlife toxicity. Such studies would be in addition to the ones described so far.

PAGANO: On the acute test, you say there are four. Can you just give us an idea of what would be four typical acute tests?

ENGLER: Acute oral, acute dermal, dermal irritation, and ocular irritation. And conditional tests would be inhalation, subacute inhalation, subacute dermal, and skin sensitization.

JOKLIK: Are there any requirements concerning concentration? How many animals would be used? And what is the scope of this sort of testing effort?

ENGLER: There are requirements for the number of animals and the number of test levels. In the particular instance of viral pesticides, probably only one dose level will be used since the type of formulations we see on viruses are usually such that the oral toxicity will be more than five grams per kilogram. If that is the case, one dose level will be sufficient.

JOKLIK: What sort of animals? Does this include both primates, or at least monkeys, rodents, and does this extend to fish and that type of animal?

ENGLER: No, primates are not included for acute tests on the formulation. Since you mentioned fish, I should include category four, which includes "fish and wildlife" toxicity on birds, fish, and aquatic invertebrates.

CURLEY: Could you explain to the group the pathogenic endpoints that result from each of these tests, regardless of the route of administration, and whether it is a per os or parenteral route of administration? You mentioned that these toxicity tests were pathogenic endpoints. I would like you to tell the group which of these endpoints you evaluate and what you would consider significant or not significant in your evaluation.

ENGLER: The tests on the formulation are primarily necessary for designing a label. On the label there will be certain precautionary statements and a certain signal word, such as "Caution," "Warning," or "Danger." Each toxicity category has a certain range within which each formulation must fall, based on the acute tests described before. The signal word and thus the toxicity category are determined by either oral, dermal, eye or skin irritation; the most severe of these effects determines the required labeling.

HOLOWCZAK: Do all these tests have to be carried out with occluded virus and free virus, or just with occluded virus? Particularly considering occluded virus, is there any requirement carried out with tests on free virus? Or do you just use occluded virus all the time? Since the fates of these are very different, apparently, in the environment and in the animal, will the requirement state what will be in the product?

ENGLER: It is what is in the product. If the product contains 70 percent occluded for 30 percent nonoccluded virus, that is what it is.

KAWANISHI: If, for example, you are studying eye irritation, exactly what are you looking for? I think what is causing some of the confusion is not knowing what you are looking for in the tests. Are you expecting the subject to die?

ENGLER: Well, the acute tests on the formulations are simply designed to reveal the properties of that particular formulation. If somebody accidentally sprays or splashes a chemical on his skin, or gets it in his eyes, we will have understanding of what might happen to his eyes or to his skin. That is all the acute tests are designed for. If the rabbit's eyes show permanent corneal opacity, for example, we should warn the user of this pesticide of the product's potential for causing blindness.

JOKLIK: Have any tests been done on the effect of these agents on plants rather than on animals or have any tests been done on nontarget insect species? For example, you may begin to wipe out bees or butterflies.

ENGLER: Although there have been tests done of that sort, I am not that familiar with them.

JOKLIK: It is not part of the requirement here, is it?

ENGLER: It is not part of the human safety requirement. This is part of the efficacy and environmental safety. A petition would not get through without it. Category four includes birds, fish, and aquatic organisms other than fish. Nontarget insects are included in the efficacy side of the application because it needs to be shown which insects are susceptible to the virus.

IGNOFFO: They have been evaluated against a representative species of non-target beneficial insect parasites and predators in addition to bees, and also against various species of economic plants.

DICAPUA: In the acute tests, either on the formulation or on the technical material, is there any titration done? Or is it a one-dose test?

ENGLER: Titration of how much you give to the animal?

DICAPUA: Yes.

ENGLER: It is a one-dose test, and we have been recommended to use "high doses," which may be as much as 100- or 10-acre equivalent of the active ingredient that is used. We felt there was no need for a gradation of exposure if we use as one exposure a rather large quantity. Essentially the same rationale applies as with the formulation, as I explained a little earlier. There would be a gradation necessary if a toxic effect is reached.

DICAPUA: So basically, it is a one-level application, and it is based on what you assume will be put on the field.

ENGLER: Right, on a 100-acre equivalent per man which then is translated into milligram virus per kilogram body weight.

KAWANISHI: I have had a request for a definition of acute and subacute.

ENGLER: Acute is obvious, that is a one-time exposure. Subacute exposure is a daily exposure for a number of days, in general, 90 days. There are some subacute dermal and inhalation tests, which are of a somewhat shorter duration, about three weeks.

KAWANISHI: I think the Federal Register says something about the subacute, that it is defined as multiple exposures for less than half the lifetime of the animal.

ENGLER: That was said at one time and we have wrestled with that definition. We now define lifetime exposure on one hand and anything less than lifetime as subacute on the other hand. The lower end of subacute studies is, as I just mentioned, several weeks to several months.

IGNOFFO: How do you select a subacute dose when you cannot obtain an acute dose response? We normally administer the highest dose we can possibly give an animal. Extremely high doses have been administered over long periods, sometimes the entire test period, with no observed deleterious effects in the test animals.

ENGLER: We are dealing with subacute tests. The definition of a subacute test has nothing to do with the dose. With a toxicant, you necessarily cannot give an animal an LD50 every day, although there are some toxicants with which you can do that. The dose, per se, has nothing to do with the delineation, whether a test is an acute or a subacute test. Again, we would like to administer as high a dose as possible; practicability and common sense are, of course, the limitations.

SMITH: In matters like this that are rather important, it seems to me that everyone would feel better about some of these tests if they were confirmed by somebody who didn't have a vested interest in producing this pathogen for us. They make a decision, but whoever is applying for the license brings the data to them and then sits down and makes a decision; I didn't understand that they actually confirm the tests themselves.

ENGLER: We make the decision as to whether the test is acceptable; somebody else, usually a contract laboratory, has performed the test for the registrant.

COLLINS: That's why I hate to be cynical, but if your job or your business or if your division happens to depend on it -- if six bugs die one day, that's going to be a plus-minus test. It certainly would be nice if there were some kind of neutral group which could at least confirm occasional key tests.

CURLEY: I would like to throw some light on this question since we are the research and development arm of EPA, the Health Effects Research Lab. It would be our responsibility, if called upon by the Office of Pesticide Programs, to test any of these viruses and/or chemical, depending on whether they have problems identified by the Office of Pesticide Programs. They have to identify the problem with the chemical or the virus if there is a question about the data as submitted by industry. They raise the question, and we as researchers will repeat that study or will commission a study outside the agency to be done to answer that question. No, we have not tested viruses in our lab. Dr. Kawanishi is responsible for our work. We are not in the testing aspect of the problem now.

DOERFLER: One of your clauses written on the blackboard is to test for invasion and persistence of virus in the test animal. I'm not connected with EPA at all, but I personally would feel somewhat more satisfied if this were rephrased to read, "invasion and persistence of virus genes" in test animals.

JOKLIK: We are coming to that. Considering another aspect of this, we now have a reasonably good idea of which tests and standards are currently applied to the formulations of viruses that are being considered for licensing. I wonder if we could focus now more on the actual viruses in these formulations.

Now, viruses, as we know, can cause two types of effects on cells. First, viruses can cause effects because of the non-nucleic acid constituents that they contain. We all know that inactive viruses can affect the cells into which they are taken up. Viruses can affect their host cells without their nucleic acids expressing themselves at all -- the toxic effect. And I think this sort of effect could be tested along these lines, provided that Dr. Harrap's suggestion is taken up, namely, that the virus be tested in an optimal diluent or suspension medium, rather than in whatever formulation is available.

The second effect that viruses have is due to the way their nucleic acid expresses itself. This was alluded to by Drs. Doerfler and Stollar, and was not addressed at all in these tests here. I wonder whether we could have some discussion on what sort of tests might be applied in order to determine how these viruses express themselves, and the genetic information that they contain. They probably do not express it completely, otherwise there would be overt signs of virus multiplication in nontarget hosts, which apparently have not been detected yet.

But to what extent can these viruses express the genetic information that they contain with resultant adverse effects on their host cells? One earlier presentation addressed this type of effect, and that was a description of Meinke's work, in which he develops or probes for the viral nucleic acid. He can also test whether the viral genome is able to persist and multiply in cells. Potentially, his test is capable of saying what portion of the viral genome persists. Additionally he can test, by looking at the ribonucleic acid, to what extent a DNA-containing virus is transcribed. Can we arrive at any sort of unanimity, any recommendation that would say that this type of test should be applied to the viruses, and if so, in what systems, in what cells, and in what possible animal organs should this sort of test be performed? Obviously, one would not be able to do as complete an analysis, but one would have to select a certain number of cells and the number of animals.

MEINKE: Primarily, we are contracted to look at mammalian cells. I decided to look at human cells. Of course, this is only one species and would not answer the question as to what is happening to other wildlife forms, like fish or unrelated insects, such as honeybees. You could do these tests ad infinitum and it could get ridiculous.

I do think it would be a good idea to test certain selected species and maybe a representative from the major families. In all likelihood, what we might find is that the genomes are rapidly degraded in these cells. If we found this, I think it would answer many peoples' doubts and inhibitions about using these types of vectors. If the reverse were found and sequences persisted, I think we would have to proceed perhaps a little more cautiously. I do not think you want to make it too rigid because you would have to test too many species. The obvious benefits for these compounds are very important to the economy. I would hate to see an unduly delay, but once again, one has to consider the safety factors.

HARRAP: Are you going to look for possible integration of pieces of baculovirus DNA into the DNA of a target host cell, because we know so little when discussing anything about replication of these viruses in a sensitive cell system? I wonder if you are going to start there?

MEINKE: That would be the obvious beginning. I guess it all boils down to the fact that we know very little about the biology of these viruses. There is strong evidence that in part of its normal replication, SV40 genes are inserted into the host chromosomes. It is a normal replication procedure. We do not know if this occurs in baculoviruses or not.

HARRAP: There is another point, which might be a side issue. In the formulation, you have chemicals, stickers, UV screeners, and so forth. Furthermore, when you put the virus out in the environment, there is the effect of ultraviolet light (which is one of the inactivators that the agriculturalists worry about) to consider, which might itself induce mutants. Perhaps we should think about this.

JOKLIK: Should we concentrate on arriving at a recommendation of a test that should be carried out? I agree that it is impossible to do everything. But it does seem to me that the time is right for trying your type of test in three or four different types of cells. If the result is positive, one would then have to go further to see whether the result is adverse. There could be a bit of baculovirus DNA in human cells without any harm being done. One would, first of all, like to know what actually happens. We are in a position where we can acquire some solid information concerning this aspect, and I think one has to start somewhere.

COLLINS: One possible approach is based upon some preliminary work that Dr. Harrap mentioned, which was the question of the persistence of the virus in the gut of mammals that have been fed test material. I forget whether it was a formulation or the active agent itself. The data indicated that for at least ten days or so, virus had persisted in this tissue.

It seems that this would be one approach that would fit in very well with the safety testing that has already been done, and the animals that are being used for these tests could be processed in this way. If the virus does persist in these animals, what sort of immune response occurs? This would probably be much more relevant to the worries that are obviously in the backs of our minds. It would also fit in very well with what is being done already. I do not know if Dr. Harrap or anybody else has any more information on this type of approach, but I think that might be one kind of test that would be very easy to do and that would have a great deal of relevance.

ENGLER: In my presentation, I did not go into details of the way we would like to see these tests done. In fact, we are requiring this type of interim sacrifice of animals which were exposed to an acute dose in order to determine the fate of the virus. This is what I meant with persistence, inactivation, and degradation of the virus in the animal. Some of these considerations are already included in what is listed here as a category three test, or acute and subacute testing.

In certain animals, virus has persisted in the gut for some time, although I am not aware that it has persisted for as long as Dr. Harrap has been reporting. On the other hand, this may reflect a difference in the detection methodology that was used then, and the one which is used now.

PARTICIPANT: (Inaudible.)

IGNOFFO: Dr. Fields asked a question concerning the fate of virus in vertebrates and humans and also about the detection level. In a study using rats, Heliothis virus was fed via stomach tubes at a rate of about 20 million LD50 units per rat. Animals were sacrificed after various periods of time, and control tissue was spiked with virus to determine the basal detection level.

The animals were sacrificed at zero minutes, at ten-minute intervals up to an hour, after two, four, eight hours and then at twenty-four hour intervals (up to 264 hours). Blood, urine, various tissues, the stomach, and the alimentary tract were all bioassayed for presence of infective virus. Using this technique, we could detect about 100 PIBs or about 10^3 virions. We feel we can detect about 200 virions using a modified technique and a large number of test animals. Using the base of 100 percent activity at zero minutes, about 70-80 percent of the original activity was inactivated or voided from the intestinal tract after 2 hours. After about 24 hours, there was less than .005 percent of the original activity. After 48 hours, it was down to .002 percent, and after 72 hours it was down to less than .001 percent of the original activity. A value of 0.001 percent was close to the level of spiked-control samples. All of the tissue homogenates as well as the urine were negative for the presence of virus.

HARRAP: These were animals that were fed polyhedra?

IGNOFFO: That is correct.

HARRAP: And you were assaying, presumably, for polyhedral inclusion bodies back into the insect?

IGNOFFO: No. We were able to detect infectious virus, presumably as free virus particles, infectious viral subunits, or inclusion bodies.

HARRAP: In larvae by feeding?

IGNOFFO: Yes. In fact, we established a temperature-stability curve for virions by using our sensitive neonatal assay technique.

HARRAP: So when you fed back, how were you certain that you were in fact assaying free virions? Did you expose the animal to polyhedra and then take tissue samples?

IGNOFFO: Yes. We were sampling for viral activity per se that would include polyhedra as well as any other infective unit. The point I wish to emphasize is that we were testing for the presence of any infective entity.

HARRAP: Whether the infectivity was from freed virions that might have been freed in the gut lumen, or whether it was from occluded virus? There is a slight difference there between what you have done and what I have done, in that we assume (I think it is generally agreed) that polyhedra will not infect cell cultures. You need free virions to infect cell culture. Of course, I was taking tissues from the animals and putting macerates derived from those tissues back into cell culture and apparently getting infection. So presumably, the polyhedron dissociated to put free virions in the gut of the animal. I think I would not, in my system, detect any infectivity due to polyhedra.

PARTICIPANT: You said you could detect 200 virions?

IGNOFFO: It becomes a numbers game when bioassaying down at a lower level of detection. I am basing the number of virions we can detect on estimates per inclusion body. There are about 25 virions per inclusion body. Thus, we can detect an end point dilution of about ten inclusion bodies (an LC50 detection level is 3 to 5 times higher).

ULVEDAHL: I have a couple of questions for Dr. Meinke. Can the test that you are developing be performed on a more or less routine basis? How long does it take to perform one of those probe tests? And what do you estimate the cost to be?

MEINKE: It is very difficult to estimate costs or time because every virus that you test probably has different probe characteristics, etc. I have personally only been involved in this since last November, and we are working with *T. ni* cells 368 and *A. californica* virus. I have approached this cautiously; I wanted to work up a quantitation system first, and we now have a very good plaque assay in the lab.

We are now to the point where we are trying nick-repair of the viral DNA. To infect cells to get enough cellular DNA, infecting maybe a hundred cultures or so per virus, extracting the cellular DNA, and doing appropriate control reactions will be necessary. As soon as we get going we can probably assay a virus every four or five months. Of course, if someone gives me purified DNA, I could probably do the whole experiment within four to five weeks. But now we are starting from scratch, starting our own tissue culture and preparing our own virus; it is very slow and tedious.

JOKLIK: Dr. Meinke, which DNA is in short supply, the viral DNA, which you would then label, or the larger, cellular DNA?

MEINKE: The viral DNA.

JOKLIK: But that should not be such a problem because you could get a bit of formulation, purify virus from it, and probably make a kilogram without much trouble. There is plenty of this material available.

MEINKE: Let me point out that I have done all my work in vertebrate virology, which is really quite simple compared to the invertebrate virology. I point this out because for a vertebrate virologist working with SV40 to get a virus yield of 10^9, or whatever, per milliliter, to go to invertebrate virology, and get 10^4 or 10^5 infectious particles per milliliter is a whole new ball of wax. For technical reasons, I had decided to do my first experiments with nonoccluded versus occluded virus. In working with nonoccluded virus, low titers are involved because most of the virus, at least in the _T_. _ni_ _Autographa californica_ NPV system, is, I believe...

JOKLIK: But this is not the point. The reason for the infectivity, why the titers are so low, is not because the assay system is inefficient.

MEINKE: That is right.

JOKLIK: So you are interested in having the nucleic acid, which is plentiful.

MEINKE: Well, if you have only 10^4 and 10^5 infectious viral particles per milliliter, there is not much nucleic acid present at all.

JOKLIK: But that is a lot of particles and a lot of DNA, isn't it?

MEINKE: No. It is a very low number, and there is very little DNA.

JOKLIK: I would like to have someone explain that to me.

KNUDSON: May I make a comment and perhaps ask a question of other people who have worked with these viruses in cell culture? It has been my experience both with the _A_. _californica_ and a number of the NPVs not to find titers of 10^4 per milliliter in infected cell cultures. I think a fair figure might be more in the area of 10^7. Would anyone else like to comment on that?

GRANADOS: This is routinely seen in our laboratories with _A_. _californica_ virus and _T_. _ni_ cells, and in other cell cultures, and the same thing holds true for entomopoxviruses -- you get fairly decent yields in the neighborhood of 10^7 PFUs per milliliter.

PAGANO: I mean what everybody's talking about, what Dr. Meinke is getting at, is that infectious virus is not the point, it is the particle to infectivity ratio that we are trying to get at. What is that?

KNUDSON: As a matter of fact, I have some experience with that using a direct particle count, and I came up with a figure (this is of a purified virus prep) in which there is a sonification step involved, so I was not only measuring my particle to infectious ratio, but the effect of my purification. We came up with numbers in the neighborhood of 200 to 300 particles per infectious unit. Dr. Summers has done some direct calculations, and I believe his figures are a little bit lower, but that has not been taken into account.

SUMMERS: That is just because we do it a little different way. When we made our calculations they were not significantly different from your results. So we considered our calculations close enough.

PAGANO: If it is a super-coiled DNA, then you are in a very good position to purify and recover with very good efficiency (even directly from infected material). Then you could go on with the labeling.

I would like to help Dr. Meinke clarify his point. You have to divide this into developmental aspects, but once he has the tests running and he has the labeled probe, then he can do this test in a matter of days under some circumstances. We do this with Epstein virus routinely. We accept samples from all over the world. If you want the complete percentage of the genome that is present in the tumor tissue or whatever you are analyzing, you may have to carry on the test for several weeks. But if you simply want to detect presence of viral DNA, then you probably will have an answer in two days.

The tests are strictly controllable; there is no real ambiguity about them, which is one of the strongest advantages.

There is one other point that keeps coming up about integration. I think the integration is very interesting as a research problem, but if you do not find evidence of persistent viral DNA in the noninfectious systems, which is what we are concerned about, from probe studies, then you know not to look for integrated sequences because you cannot even identify them. The more difficult question of deciding about integration does not have to be approached; it is certainly not the practical level if you do not find anything persistent.

MEINKE: An ideal way of doing these experiments is, of course, doing a time curve in which you take the zero time and find out how many genome equivalents you have put into the cell. Then you just wait and see what happens to it. Let's say within five hours after infection you no longer find viral genome sequences, then you have a pretty good indicator that the host cell is going to degrade it, and there will be no problem with it.

SMITH: I would like to return for just a second to the original discussion. What kind of guidelines could be applied to where these tests are directed? I think consideration of these guidelines and their recommendations should include the biology and actual likelihood of exposure of these agents to whatever is involved. And, of course, I think that where you have to start is the insect cell itself. In the homologous system, if the gypsy moth is being used, what happens to the virus DNA or nucleic acid in the insect or the gypsy moth cell? The second test system that has to be examined involves the predators or other natural ecosystems that will be exposed to that virus, sometimes in a large amount.

Finally, you have to look at either man, vertebrates, or other indicator systems that indicate whether man is at risk. I think that you are obviously dealing with limited dollars and limited time, and in some cases, levels of detection that are relatively sensitive or insensitive. I think these are the sort of priorities I would recommend.

FIELDS: I thought it might be worth making one point explicitly. The first issues, as they were outlined, essentially summarized what we have all heard, underlining the safety tests as they have been done, which have worked quite reasonably to date. As an editorial aside, I think all of us have been impressed with the thoroughness with which the work has been done and the development of this as a system and the enormous amount of data that exist. Essentially, the issue that has emerged in the last few minutes is trying to discover how to make feasible more refined probes. Then the distinctions have to be made about immediate feasibility and immediate applicability to the issues that are coming up in terms of safety versus the high priority items for developing the kinds of further, more refined tools for guidelines.

As Meinke presented the data, and as the facts were discussed, it was clear that there is no absolute timetable. But it is also clear from all of the comments and concerns that this should be an issue to develop in terms of immediate priorities, instead of a separate issue from the immediately applicable standards and guidelines. I think it is very important to distinguish what is the immediately practical and real first line of testing, and then we will attempt to refine the issues of safety.

The first issue that certainly involves many of concerns in terms of persistence, integration, and human effects is the DNA hybridization techniques as they are going to have to be developed over the next year or two.

HOLOWCZAK: I just want to say that if you are going to define a test, then you are going to have to say something about the kinds of cells that are going to be used. We have already heard that some of these insect cells possibly carry latent viruses. We do not want to get into the bag of using those cells in trying to super-infect it, so to speak, and getting a result that may be meaningless. Perhaps, since there are people here who are experienced with these cell lines, they should attempt to define which cell would be best for use in such tests.

What do you infect with occluded virus, the virus freed from the occlusions, partially degraded virion, perhaps nucleic acid and protein, or maybe free DNA? All those things should be used to complete an adequate test. Or should one actually look in the gut of the animal to find out how the virus is degraded? You might end up with some unusual intermediate which would gain access to animal cells where something else may not.

I think these are all very important considerations in defining the tests that could be used. But I do not know if you are going to find anything out from simply throwing occluded virus on cells. From what has been said, these things do not get into the cells. But apparently, labeled virus can now be grown; so you could do the straightforward kind of uptake studies to find out if nucleic acid even gets into the cells, before you begin hybridization, perhaps, and then go to hybridization studies later. But I think you have to define which cells you are going to use and how you are going to apply the virus or subviral particles and make the nucleic acid.

RAPP: There are really two problems. One involves what should be done now in the light of current information, and the second concerns what should be done in terms of further developing the research side; obviously, the two are related. But this discussion has taken the turn that nucleic acid hybridization may be the cure-all for everything, which it probably is not. It would seem that one of the problems that has arisen concerns the origin and genetic make-up of some of these viruses, about which relatively little is known. That is obviously an area of fruitful further research.

The second problem is to determine whether these viruses are potentially dangerous in nontarget tissues. Apparently, this is a very large problem. And here molecular techniques are valuable, but so are those techniques that measure RNA, or say, the synthesis of viral protein using the very sensitive methods that have been developed lately, such as the radioimmunoassay. It ought to be very easy to develop, as Dr. Summers has already described, highly sensitive immunologic probes. If you do it Dr. Meinke's way with a time-curve analysis, it is a very nice intellectual exercise and a nice research problem. But I don't believe it is really what the EPA needs for testing viral pesticides.

I think the tests have to be simplified in a rational way, just as the number of cells and the numbers of animals have to be simplified. Otherwise, the burden gets so large that the program stops in its tracks, not only because we do not have the funds, but because there is no available manpower or time for a great number of tests. One part of the problem that this type of meeting or any kind of committee meeting like this should address is minimizing the amount of testing required, while maximizing the information and the effectiveness of the tests. I think this is one area which should really be considered by this group.

The next area is research of the future and the kinds of priorities given to it by various funding agencies. Many of those items are likely to come up when you find out that a lot of the tests you would like to do and need to do cannot be done because the basic biological or biochemical information is not available.

STOLLAR: I would like to make just one technical, but important point. In looking for the expression of these viruses in nontarget cell lines (work in vitro), I think it is very important to pay attention to the temperature at which these experiments are carried out. I think it is unlikely that these viruses will replicate or express their genome at the normal temperature for mammalian cells. Perhaps if mammalian cells are used when these experiments carried out at say 34 to 32 degrees, a great deal of attention should be directed to using various poikilothermic cell lines at which these viruses would be more likely to replicate or express their genome.

COLLINS: I think there is one problem with that. If we are using in vitro tests, particularly in mammalian cells, as valid models for what may be happening in the field in a natural situation, the viruses are going to have to adapt and replicate at the body temperature of mammals.

STOLLAR: But I think to begin with I would look at these temperatures where it might be more likely, and then probably move up to higher temperatures.

SUMMERS: I would like to get back to what Dr. Rapp said, and what Dr. Meinke has been trying to get at -- what cells should be tested, and what would be a minimum acceptable first attempt?

JOKLIK: It is true that the predator pathway is harmed. Nevertheless, it seems to me that there is no question that if you spray these viruses from airplanes, that irrespective of any other way of acquiring them, man will be exposed to them. What I would recommend is that the minimal test focus on a human cell. I think the fundamental thing one wants to know is, what is the fate, the expression, and the effect of viral genomes on human cells?

SUMMERS: I agree with you, but at the same time I disagree. These are invertebrate viruses, and although I consider myself more important than anything else in this world, I think that the possibility for crossing over in invertebrates is much greater than in man. Now, I agree, we need to go ahead and test the human cell system, but attention must not be drawn away from another area that is potentially more realistic in terms of potential infection.

RAPP: The diversity of insects in any given area to be sprayed potentially causes problems. It would be hard to know where to limit testing procedures. I suspect it might be a long-range thought, but I am not sure how critical it is in an attempt to fundamentally protect the human, and perhaps, other economic aspects of our system. If you tried to protect the whole ecosystem, the amount of testing that would be involved would be beyond the range of anything that we could visualize.

SUMMERS: That was not the implication of my statement. If you start with one human cell line, then you can do the same for one or two other nontarget invertebrate cell lines.

HARRAP: This is perhaps an unnecessary reminder, but it is worth saying at this point, that although we may be applying these viruses from airplanes, large levels of virus can occur naturally in a forest area. Any forest management authority, and there are some representatives in this room, will tell us that if there is a large pest situation with a natural epizootic of these viruses, there are vast quantities of polyhedra in the atmosphere in the forest anyhow. So it is not as though we are doing something totally alien. Of course, I realize all the risks and ramifications.

VOLKMAN: Should port of entry be considered? Do you use lung cells or gut cells?

JOKLIK: That is an important point. Could I just come back to Dr. Harrap's point, which is, of course, a very important one -- would you extrapolate from that, and I want to play the devil's advocate, seeing that large amounts of these viruses do occur naturally all the time, and man has been exposed to them for considerable periods of time with no ill-effects, would you regard that as some sort of argument that suggests that these viruses really do not have an effect on man?

HARRAP: It is an argument I consider frequently. Yes, I think one can base some elements of a case on the fact that there have been a lot of people involved in veterinary and human virology, and if there had been some clinical effect of baculoviruses on forest workers, for example, that might well have been detected by now. I would not like to put undue weight on it, but I do not think we should lose sight of it.

JOKLIK: I think it is an excellent point. The follow-up question was about what type of cell to use. Would you like to expand on that?

VOLKMAN: It was just a thought.

GRANADOS: This is somewhat related, and I wanted to come back to this problem -- if we are going to understand what is going on in a vertebrate non-target cell, we obviously are going to need an excellent base line; we have to know what is going on in the homologous-susceptible system. I wish to make the point that we still have a long way to go, because except for a small handful of NPV-type baculoviruses, we do not have good homologous systems with which to study these viruses. We have no good system for the gypsy moth, and the same thing can be said about the tussock moth. I am talking about Autographa californica NPV, T. ni NPV, and Spodoptera NPVs. No susceptible insect cell lines have been found for any of the granulosis baculoviruses. So we definitely are going to run into difficulties when we look at the fate of granulosis genomes in vertebrate cells. What is going to be our homologous system, what is going to be our baseline for study?

KNUDSON: I would like to re-emphasize that point, which was one of the points I was trying to make earlier in my presentation on the in vitro specificity, or host range of these agents. I tried to put forth some sort of methodology, some sort of mechanism by which we could assess the level of sensitivity in the tests, and at the same time, assess the level of susceptibility of a given cell line, for a given viral agent, in which I have only looked at six different, or apparently different, NPVs in a handful of cell lines. The problem really seems that we do not have the baseline data in terms of *in vitro* specificity or host range for lepidopterans, let alone the mammalian or vertebrate systems. First, let's look at things at one level of sensitivity in terms of the detection of the viral-replicated effect or event in the cell. Then, as new methodologies become available, as they seem to be coming available, we can refine and improve our sensitivity in our check, but we do not have the baseline data yet.

IGNOFFO: I want to get back to the original question you posed. What seems to be coming out of this discussion is that reassociation kinetics is the best available approach. Do you mammalian virologists agree with that?

MEINKE: No.

IGNOFFO: Well, then, can you tell us what technology can be used to advance the detection levels beyond those we have dealt with in the past, especially as it relates to a viral genome in a nonhomologous host?

MEINKE: I do not want to imply that just because I gave a talk on DNA reassociation that it is the very best technique, and I hope it did not come across in that manner, but it is highly sensitive. You can certainly detect one viral genome equivalent per cell. Not too many immunological methods can detect a virus particle per cell. Even though it is a sensitive technique, I do not want to say it is the only one. We have experts here in other fields. Let them discuss their areas of expertise.

JOKLIK: Would others like to join you?

FIELDS: Well, since I am not doing the hybridization, I would disagree. I think you are talking about measurements of DNA and RNA and protein, and among those there are a variety of systems by which a selected number of techniques have ultimately become very powerful. Others have more experience with some of them than I do. But I do think you have to have a focus in developing DNA hybridization. I think you have to develop a hybridization analysis for RNA, and then I think when it comes to proteins there are very sensitive measurements for the gene products in terms of radioimmunoassays. I think there is enough precedence and a variety of systems that variants of those three techniques are the ones that should be developed and made available relatively quickly. There are a lot of techniques that we have used and become familiar with, but those are the two in particular, of hybridization and radioimmunoassay for nucleic acid and proteins, that have turned out to be the most sensitive for answering this particular question of small amounts of either nucleic acid or protein.

RAPP: The plea I was trying to make was to use them all, because it all depends on what happens when the nucleic acid gets into the cell. If, in fact, it is cut and does not do anything, it is going to be very hard to detect. But suppose it is transcribed -- and there may be much larger amounts of RNA there than in the original DNA that is there -- if that is translated there may be large amounts of protein compared to either the RNA or DNA. So an amplification would be possible.

It is impossible to predict, I would think, in advance in these abortive systems, because one could give examples of any one of these circumstances as to which technique to use. So the best thing is to simply utilize, for the three basic compounds one would be looking for, the most sensitive technology, and for that, nucleic acid reassociation kinetics and the radioimmunoassay would be the techniques of choice. But I do not think one would or should exclude any of the other tests.

FIELDS: I agree with that completely.

JOKLIK: Was the technique that Dr. Meinke described, pioneered by Dr. Pagano, for finding the Epstein-Barr virus genome? That is an extremely powerful technique. I do not know whether I would subscribe to the fact that certainly both DNA, RNA, and the radioimmunoassay for protein should be looked at. Would you subscribe to that, Dr. Pagano?

PAGANO: I was just thinking about the proteins and the RNA. There is a problem with the proteins. There is one point that has not been made explicit, and that is that we are really concerned about a nonproductive infection. Let's just underline that, so if you start with the basis of a nonproductive infection, the DNA, it may not be expressed in any measurable way. For example, in the Epstein-Barr virus system, the only product or protein made is a nuclear antigen. That is an extremely sensitive test in the sense that if you can find it with a simple immunofluorescence test that anybody can do, that is fine, but the problem is that you have to have that protein identified first. And so, whereas you can look for the viral DNA directly without first having to find which proteins that are novel are made in these cells, this line of investigation has got to be with the DNA; the RNA would follow close behind. With luck, there may be a new protein that you can identify and also detect. Then you have to select which is the simplest and most sensitive. But I think it does start with the DNA.

JOKLIK: Now I absolutely subscribe to that. In most transformed cells, capsid antigens do not express themselves. Nevertheless, with these viruses the only radioimmunoassays that you could devise would be for the capsid proteins because there is no way of making antibodies for nonvirus proteins. DNA and its expression, namely, RNA, are the two basic techniques. The radioimmunoassay may or may not be useful. I agree that it is most probably amplified. But at the moment, the only way of doing it would be amplification of a protein, namely, a capsid protein, which is most likely rot to be made.

RAPP: I do not agree.

JOKLIK: Well, in some systems, certainly that would be true, but maybe there are exceptions. But it would be hard to make the antibody for the right protein.

FIELDS: It might be worthwhile to point out that the fluorescent antibody technique which was mentioned is very sensitive and should also be developed. It also would be a useful other probe for expression in terms of proteins (both capsid and noncapsid proteins). Could someone comment -- it is mentioned on page 181 of Baculoviruses for Insect Pest Control: Safety Considerations that "The fluorescent antibody technique, for example, or other sensitive methods, must be applied to a selected number of exposed tissue cultures to determine if viral antigens are subunits that would not be detected by bioassays where produced." The fact that it may be an easier and more sensitive, in terms of current knowledge, technique to use would be one point, but the second is to ask if any of this has been followed up or whether any aspects of this have been developed.

JOKLIK: It is not very sensitive, though. With the RNA, we can certainly detect one RNA per molecule per cell. But for the fluorescence you need many protein molecules.

FIELDS: Well, the main point was, historically, if one is looking for evidence of persistence in a nonproducer way, as with the SV40 with adenovirus, it has been a useful tool. Dr. Rapp, do you want to comment on that in terms of taking immune sera, and using this as an assay for a protein you may not have your hands on? The T-antigen is really a good example of one that has been very difficult to get in terms of the ways we have been talking about, in the quantity that it is detected.

RAPP: Of course, the fluorescent antibody tests are not as sensitive, but you do not need the highly purified probes that you need for the radioimmunoassay. You can take a much cruder serum made against a large number of proteins and start a general hunt, if you would like to call it that.

JOKLIK: I think it is undoubtedly a very valuable accessory item.

RAPP: That is right, and the only reason I was disputing the point with you before was that with all the small viruses the capsid proteins are not made in transformed cells, but with the larger ones, such as the herpesviruses, it turns out that in many cell lines they are made.

KNUDSON: I just have one question here I would like discussed, and obviously, I am not acquainted with the nuances of the significance of certain of these tests. But what if you do find a genome in the cell, what significance do you make of that finding? Would you care to discuss that, please?

JOKLIK: Dr. Rapp, would you like to, because you raised the question of carcinogenicity and teratogenicity?

RAPP: Well, I think that if it is demonstrated that one of the baculoviruses can exist in a persistent form, whatever that form may be in mammalian cells, especially if they are human cells, then that would put quite a different light on safety testing and on whether one is prepared to utilize that particular reagent in a large-scale testing phase.

Now, it is hard to know what it could do to human cells. For one thing, not knowing the biology, I would like to know whether these viruses would have an effect on neural cells. Could they possibly go latent in some cells? Could they be activated later to cause some unknown disease? I like Dr. Harrap's point that there are really no good examples of these viruses causing disease, apparently, in man. But I also have to point out that these viruses are going to be selected after passage in subculture and that may change some of their biologic properties. I am convinced, incidentally, I take it as an article of faith, that the DNA of these viruses will integrate in certain types of cells. They are, in effect, circular molecules, double-stranded DNA, seeming for all the world like SV40. There is no reason whatsoever, given the right conditions and the right cell, that they would not be able to integrate their genomic material, or at least a portion of it. If they do that and convert the cells to malignancy in the process, then I think this should be real cause for much more testing and much thought before using such an agent in a biological system.

FIELDS: Well, taking off on Dr. Rapp's last point brings us into the other category, just briefly, of long-range needs. What Dr. Rapp is essentially saying is that we still do not know how to formulate the questions because the biology and genetics of this system remain at a very early age. Now that does have to be separated from the immediate practical questions of safety testing and what has to be developed very shortly in terms of immediate applications for more sensitive probes. But it cannot be separated from the fact that when you begin to raise these questions and ask what happens if you get a test that gives a certain result, in order to really answer the questions that will be raised by anything that turns out positive, there is no currently available information in terms of the biology and genetics to use for forming the question.

So that leads to another type of recommendation, a long-term one, that whatever the political realities, there really needs to be an urging for more long-term support and attention to the biology and genetics of model systems in this type of agent.

SMITH: What I thought I might do is discuss some thoughts on these proceedings in three categories. First of all, consider predictions as to how we might identify some trouble spots. What kinds of things might we envisage would cause problems? And the top of that list is activation of latent viruses in invertebrate or vertebrate cells. For example, one might get by infection of these viruses a new virus which comes out which you do not know anything about, which then could cause problems. This possibility has ample precedent in mammalian virology.

The second category is the establishment of persistent infections in invertebrate or vertebrate cells where you infect the cell with a particular virus and that virus persists for a long time and causes changes that you talked about earlier.

The third category is genetic recombination between related viruses that give rise to new viruses with altered host ranges and other adverse side effects. Dr. Fields alluded to this. That is, if one considers a polyvalent material, in which you spray with closely related agents, do those agents interact with one another to give rise to a new agent that could have new and unexpected results?

Attenuation is a potential problem. That is, you want the virus to kill the organism against which it is directed. Well, in the field or in preparation of the virus you may have the loss of virulence of this material and wind up with a very expensive but useless reagent. The other side of that coin is super-virulence, where you now have, by the propagation or in vivo passage in the field, a virus which has very unexpected and extremely virulent reactions.

And lastly, in this category, I think it is very important to pay attention to those viruses which were termed yesterday, "invertebrate-associated vertebrate viruses," such as the old category of arboviruses, etc. What is the exact state of the cell you are dealing with as far as Sindbis and Semliki Forest virus, and these other viruses which we know grow in insect cells?

The next category is recommendations for future research. At the top of this list is to characterize the DNA by two methods, restriction enzymes and hybridization. I think one of the most powerful tools now available in animal virology is comparing various viruses by their restriction enzyme patterns. How does virus A differ from related virus B in its pattern of restriction endonuclease fragments? This can be very, very useful.

Second, to prepare mutants so that you can have markers which you can follow in a vaccine, or in this case, a deliberately administered virulent virus. These can be temperature-sensitive or host-range mutants, which would be useful.

Third, to define the replication of baculoviruses, particularly. I am encouraged and impressed by the amount of material that is available on baculovirus structure, but I am also quite concerned that very little is known about how these viruses replicate and many, many details of the replication cycle are completely unknown.

Fourth, these viruses, as much as possible, have to be plaque-purified, biologically purified as much as possible, so that you can work with defined agents, reproducible from one laboratory to the next, dealing in viruses in such a way that they are well characterized.

Then, fifth, to continue the development of tissue culture systems -- and this will have a variety of ramifications -- first of all, for virus production itself. I think most everyone agrees the ultimate production method for these viruses will be in tissue culture. While, as we understand now, baculoviruses do not grow that well for field administration in tissue culture, the nonoccluded virus is not the advantageous virus for spreading in the field. So you need to develop some type of technique for preparing occluded viruses.

Second, tissue culture is very useful for virus purification. When you want to use a defined agent, you want to get it away from all the other viruses that may exist in the field, since the squashed bugs that we have heard about can have other viruses. You get away from crude preparation by growth in tissue culture, and this requires further development of tissue culture systems.

And last, tissue culture needs to be continued to develop, to really understand the growth cycle of baculovirus and other invertebrate viruses. The last thing in this particular recommendation for future research is that I am impressed with the production of polyhedrin and granulin as markers of these particular viruses. They seem to be rather unique and biologically interesting proteins.

Second in this category is to test in a wide variety of cells. I have already alluded to the fact that I think invertebrate cells, poikilothermic cells, and mammalian, specifically human, cells, should be used.

And last, most of the tests for replication that I have seen so far applied have been rather unsophisticated. When one looks for replication in new cells, one necessarily has to look for production of new virus. That is the ultimate test for replication and this almost always implies the use of labels. Could you uniformly apply tritiated thymidine or other nucleic acid precursors to test for the appearance of new virus?

Most of the tests I have seen applied have used the sole criterion of new polyhedra formation. You look for the formation of polyhedra in bat cells or other cells; however, polyhedra formation may be a host-specific response in that you may find these viruses only form polyhedra in insect cells. And if you put the same virus in many other species, do these cells, in fact, form polyhedra? Since we know that, at least it is assumed that polyhedrin is a virus-specified product. The polyhedron is a relatively complicated structure and it may be there is not the proper host material available for its synthesis.

RAPP: I would just like to raise one other issue for future research because I think it bears very heavily on the practical use of viral pesticides. I have not heard much about it except for the one study that Dr. Ignoffo mentioned, and that is the whole problem of resistance in the field. If, in fact, insects develop resistance to chemicals, and they obviously do, and they are like other biological materials, then clearly, they are going to develop resistance to viruses.

The question that arises is whether a combination of chemicals would prevent that phenomenon. I believe the Forest Service has been spraying and may have some practical field experience, although I suspect it will take some years for development of resistance, as it took Neisseria some years to develop resistance against penicillin.

We know from a lot of other experience that it is bound to develop, and the idea is to try and abort it, if possible.

JOKLIK: Would any of the forestry people like to respond to that?

CUNNINGHAM: In the late 1930's, Diprion hercinia was an important sawfly pest from Canada. It was accidentally introduced in Europe, and it came over here without any of its parasites or viruses. In fact, the spruce in eastern Canada was in great danger. About 1939, a new virus was introduced along with parasites from Europe, and it spread throughout eastern Canada over a period of 10 to 12 years. We still have it, and it is an absolutely perfect example of biological control in that we still have the pest at low level, it is of no economic importance, and we still have the virus at low level. There is no indication that there has been any change in the resistance of the insect or the virulence of the virus over a period of approximately 27 years. From the point of view of resistance we are probably looking at a short time because the other viruses we have been working with for only seven or eight years, and there is no indication of change of the LD50 of the virus we are now working with -- and there would be two or three other species.

HARRAP: In our discussions we have worried about the potential of these viruses for mutation and the frequency of it. Of course, insect populations may change, but the viruses themselves might also; so that if the insect developed resistance to the original virus, it may not be resistant to a genetically altered virus. It is rather speculative, of course, but one might be able to develop this idea and genetically engineer a virus to overcome any resistance.

SHAPIRO: The gypsy moth was brought to the U.S. in 1869, and in 1906 the first sign of any type of virus disease was found and it was classically called "Wilt," described adequately by Glasser and Chapman. This virus occurs naturally in populations and this virus is found literally every place the gypsy moth has been.

When the populations get to be of a high density, at some point in time, there is a total crash and inevitably, this virus, this nuclear polyhedrosis virus, is the cause of it -- and this is 1906 and it is now 1977. The Forest Service is trying to register this virus for usage, aware that it is there as a natural part of the ecosystem, but instead of allowing nature to take its course with these very high population levels existing, to try to bring the virus into the ascending populations to reduce the ultimate damage.

GRANADOS: At this time, in regard to the spray programs against forest pests, and in regard to the possible combination of virus insecticide spray programs, I would like to ask Dr. Rozee from Dalhousie University if he would please comment on some of the recent findings he has made in the spraying program in parts of Canada.

ROZEE: I feel a little awkward because I didn't even know when I came to this conference that you used emulsifiers with virus pesticides. I just learned that an hour ago, so I now think that perhaps what I have to say might be pertinent. Could I show a slide?

We became interested in emulsifiers when we looked at a cluster of Reye's syndrome children and found that the geographic distribution of these children generally followed the distribution pattern of an aerial spray program. There were only a dozen of these children but bear in mind, one would expect from the U.S. experience to only have three or four kids with Reye's syndrome in our population. We had fourteen in Canada, which was a very high incidence.

So we became interested in the components of the aerial spray and developed a rather difficult animal model, which basically looked at the effect spray exposure of mice had on the virulence of EMC virus. We have evolved from this to the certainty, at least in our minds, that the insecticide component itself was not really causing the increased mortality in our mice. The model we use now is based on tissue culture (Table 1).

First, I will explain the method and then we can look at the results. It is a very simple experiment. You expose cells (the cells we used were VERO, human kidney, LLC-MK$_2$, L-929, and HeLa) for a period of a few hours to Toximul MP8, which is one of the more favored emulsifiers for pesticide aerial sprays, and then you subsequently wash the cells and infect them with vesicular stomatitis virus. Later I will show results with other viruses as well. You will see the control number of plaques in the top line,

TABLE 1. THE ENHANCING EFFECT OF THE EMULSIFIER, TOXIMUL MP8, ON THE ABILITY OF VARIOUS MAMMALIAN CELL CULTURES TO SUPPORT VESICULAR STOMATITIS VIRUS (VSV) REPLICATION.

Cell type	VSV plaques per culture* Concentration of Toximul ppm				
	0	0.25	1.0	2.5	10.0
L-929	50 ± 0.6	51 ± 4.5	65 ± 4.4	111 ± 7.2	218 ± 10
LLC-MK_2	21 ± 1.8	20 ± 2.6	23 ± 2.4	35 ± 3.2	54 ± 3.8
VERO	69 ± 1.8	72 ± 4.3	76 ± 3.0	99 ± 3.1	110 ± 5.3
HeLa	68 ± 2.8	196 ± 6.6	490† ± 10	380† ± 40	Cytotoxic
HK	27 ± 2.4	32 ± 2.3	41 ± 2.3	79 ± 4.9	73 ± 2.8

*The values are the means ± standard error.

†Estimate; cultures had too many plaques for accurate counting.

and the columns represent parts per million exposure to the emulsifier. You see that the number of plaques on VERO cells, for example, really does not change very much, regardless of increasing the amounts of Toximul MP8 to which they are exposed. However, if we consider HeLa cells, we find they are extremely sensitive to the enhancing effect that Toximul MP8 has. L-929 cells are also quite sensitive.

These doses of Toximul MP8 are less than the toxic levels of the emulsifiers. Many of these concentrations are considerably below the toxic levels of the emulsifier. This has all sorts of implications when you consider that Toximul MP8 itself is less active as an enhancer than a compound called Atlox 3409. We are now looking at some 14 others, and we have 80 or so waiting in our refrigerator. All of these are used widely, not only for sprays, but also for other things. I think that we should, perhaps, learn from the chemical pesticide industry, that we should look at the total package we are going to deliver, rather than just purified virus or a vehicle alone. I leave that up to you to draw your own conclusions from the data.

The next slide depicts another series of experiments, and it simply reports in figures what the previous slide showed pictorally. The slide depicts the cell type running down, concentrations used for the exposure, and the number of plaques. You can see that HeLa cells are very sensitive to the cytotoxic effect of the Toximul MP8.

Now there are two things apparent. First, different cells vary according to their sensitivity and second, the plaquing of different viruses is affected differently. This virus, VSV, happens to be a particularly good virus to work with, but Reovirus is rather unaffected by the enhancing capacity of Toximul MP8. So you have at least three important factors: the cell, the virus and the various emulsifiers.

COLLINS: Does an emulsifier have the same effect on enhancing the replication of the insect virus it is being used with? Has this been considered?

ROZEE: No, we have been talking about this during the first part of the week. We will probably look at it. Dr. Stoltz has volunteered.

PARTICIPANT: (Inaudible).

ROZEE: The commercial spray we were concerned with contained Fenitrothion. Fenitrothion is immiscible with water, of course, and it was dissolved in our formulation in Aerotex 3470 as the solvent, and then emulsified with either Atlox 3409 or Toximul MP8 in water for spraying. It took us awhile to find out that Toximul MP8, although a nonionic surfactant, is really some cut distillate, and the company that supplies it does not really know what it is chemically. We will tell them shortly what it is because we are examining it by mass spectrophotometry and gas chromatography.

ANGUS: We have never found it necessary to include an emulsifier in our nuclear polyhedrosis virus preparations.

ENGLER: I would like to make a few comments on Dr. Rozee's presentation. This is an example which demonstrates that studies done in an *in vitro* system may not always lead to results which can be used for a risk assessment without further corroboration. We have seen that a pesticide emulsifier was capable of increasing viral penetration and viral plaque formation in tissue culture. Although this is interesting, we have a long way to go to prove and conclude that these emulsifiers are enhancing viral infection in a living animal, including man. Although this is an important contribution, jumping to conclusions without further evidence should be avoided. Prior tests in animals were less than convincing. I think it is extremely important to keep this example in mind when we consider and eventually evaluate *in vitro* tests with insect viruses for the purpose of safety evaluation.

KAWANISHI: I would like to point out that there is an article in the journal, Science, pointing out the same effect with EMC in mice.

ROZEE: We made the original observation in mice and went to tissue cultures to examine these emulsifiers because it was easier and more accurate. Small differences between the emulsifiers which affect their enhancing capacity could not be measured in mice without using a very large number of mice. So we went to tissue cultures after we found the response in mice. In 1974, we published the data on mice in Science.

RAPP: I think the point might be made that while it is an interesting observation, there are a number of different ways of doing the same thing. For example, light trypsinization of cell cultures will allow certain myxoviruses to plaque much more readily. There are many examples of this type of effect. It is somewhat easy to control in cell culture and show these effects -- it is often more difficult with the whole hosts, but it is not an unusual effect and I would think it did not have any major bearing. I would be more worried about any dealing with live viruses that such compounds might, in fact, inactivate certain viruses that would have to be tested every time we mixed anything together in any live product.

FALCON: I would like to provide some information regarding the approach we utilize in applying viruses in the field in California. The main objective of our program is to utilize materials that are environmentally compatible and to avoid the use of anything we consider to be a foreign agent. We are working with waterless systems, as I pointed out earlier, because water provides potential for contamination. So one approach that we utilized is the suspension of virus in vegetable oil, cotton oil, soybean oil, and corn oil. And this is applied through very special application equipment, which are being developed. If we do find the addition of a very small amount of water, it will give us an invert emulsion, which provides much better coverage, and of course, you need an emulsifier to accomplish this. We do this using a medicinal soap, which I think many of you would be familiar with.

JOKLIK: The three questions I see are as follows:

First, do we want to recommend any additions to the procedures to be applied for testing the safety of agents that are either in use now or that are about to be licensed? Are there any immediate recommendations that we want to make? Now, obviously, these would have to be kept simple and direct, and they would have to be tests that could be carried out in the foreseeable future.

Second, could this conference provide a series of high priority topics for insect virus work, together with recommendations, for increased funding? Are there some high priority areas where we really need new information?

Third, what mechanism could be suggested to have at least the first of these listened to by the EPA? How could we have some impact on the laws and regulations as they are written? Will it be sufficient for this conference to come out with a report? Should this report be published, perhaps in a journal with national visibility, or in the form of a report or a letter? And how could we have some impact on the decision-making process?

MEINKE: It seems very apparent from yesterday and today that present testing methods for these viruses, which are probably not implicated in any disease entity in man, are probably adequate. I think what has come out in these meetings is that we should stress in whatever final report we give that if we recommend some kind of funding from EPA, it should be for basic biology related to the baculoviruses. And we should not be so concerned with the doubtful possibility that these viruses are going to be infectious to man. It seems like you just cannot answer any questions until you learn the basic biology of what you are working with, and it seems to me that should be stressed. This is an area that should be funded. The people who are working in the field and have developed it over the years have far to go.

JOKLIK: To a large extent, I agree with you. But on the other hand, we are living in an age where the public is becoming very conscious of safety. We have seen this in a variety of areas. I fear that if there is publicity without definitive information that large amounts of virus are being sprayed without any effect, that this could very well kill the program. I do not expect these viruses to have any drastic effects on the majority of the population, but on the other hand, I feel we need to take precautions to a certain extent.

MEINKE: Well, to some extent we have already taken some precautions. Dr. Ignoffo's studies and others have shown that there is probably little likelihood that the viruses do have an effect.

JOKLIK: And it is not going to be simple toxicity because the DNA recombinant research has shown that.

MEINKE: Well, I was going to bring that up secondarily. When the public becomes involved, it is sometimes not good for science; using recombinant DNA as an obvious example of an incident the public exaggerated.

IGNOFFO: I think the insect virologists, insect pathologists, or whatever you want to call us, should be proud of what we have done and how we have judiciously and prudently tried to do those tests that would demonstrate possible hazards. We will never have absolute assurance of safeness or lack of hazard. We must continue to evaluate possible hazards, using the best currently available techniques. That is why I asked a question earlier...what are the best kinds of tests to demonstrate that the viral genome is in the host and to indicate what it is doing in the host? If specific techniques are necessary and not available, then let's develop these and apply them to the most critical vertebrate system available and preferably use a system which has previously demonstrated lack of susceptibility to an insect virus.

COLLINS: In terms of steps, I think that could be put into action in a relatively short period of time. I would like to suggest two things, one of which I think could be done rather easily, very quickly, and with relatively little trouble compared to what is being done already. The other one is a bit more complicated and I imagine would be more controversial here.

The first one would be in terms of the toxicity testing that is now being done on the formulation and the active agent that goes into the formulation. I think there should be testing, and I think the state of the art in this field is now such that the most biologically pure form of the infectious agent is now testable and does not require doing any other types of tests than are already being done. It would just be another set of experiments along these lines, using that as the material being tested, and that is the first thing.

The second thing is -- this came up before, and I do not think it got the amount of attention it is due -- I think there are real questions here about the fact that, as it is presently set up, EPA accepts the data from testing organizations who obviously have a strong vested interest in the approval of these data. I think if one considers what animal virus vaccines have gone through in terms of independent testing, then it is very appropriate to recommend that if EPA, or whoever is going to be the regulatory agency, be adequately funded and adequately staffed. I do not really think, given the kinds of testing being done so far, that this would require a great deal of additional staff. In any event, that they be given the funding and staff necessary to confirm these results and repeat these tests is very important, particularly if any problems arise with this program relative to the public.

So far, only two insect viruses have been registered, although perhaps one more is close and more are being tested. So it is a relatively small number of agents we are talking about, and the experience that so far has been relatively positive and trouble-free may be very misleading. I think it is very important when talking about this as a long-range program, especially one to replace chemical pesticides, that a program be set up where these sorts of data can be independently confirmed and confirmed by the organization that is involved in regulating their use.

ENGLER: I would like to add something to the three points the chairman made. He suggested recommending topics of high priority -- maybe we should look at these topics and separate out those types of tests that are near completion, and are near the point where they can be easily used to assess the safety in a different fashion than we do at present. Alternatively we should separate out those other types of tests which are concerned with the basic advancement of knowledge on these viruses. In other words, we should red flag those types of areas that would give us an immediate benefit.

DOERFLER: With regard to the types of tests that have been discussed, it might be of interest if we commented on the type of program that has started in our country, in Germany.

As most of you are probably aware, the Farbwerke-Hoechst Company, with the support of the Federal Ministry of Research and Technology, is trying to develop these viruses, and there have been several people at universities, acting as consultants, who voiced their opinions about the most appropriate safety tests to use. I do not want to go through the details of these discussions, but I would like to express my personal opinion here. Earlier, I said something about what I thought would be applicable from the field of adenovirology, with which I am familiar and believed could be applied to this type of problem. Further, I very much support Dr. Meinke.

We have been involved a little bit in tooling up to the same type of experiment Dr. Meinke is using, and I believe it would be interesting to see what the implication of reassociation kinetics would have in this regard. I personally feel that this is the most powerful tool and the most immediate thing to be done because, as much as I agree that one should look for expression, if you do not find one genome or fragments of it, there is little likelihood that you will find its expression. I think those things eventually need to be done, but maybe the first one is to look for DNA.

ANGUS: When we decided to test a virus against spruce budworm under field conditions, we thought it appropriate to have some safety testing done. We considered doing it ourselves, and then the question came up of who would believe us since we have a vested interest in proving that what we are going to use is safe. We made the decision to take it out of the house and took it to the Ontario Veterinary College, where the work was done on a research contract. This decision was made to use this institution because of its standing and experience with the equipment.

When I discussed this with our own Canadian authorities, they were reluctant to set up a scientific group of their own. They muttered things like, "It is an old established practice that the judge and the jury shall not be the same person." Now, I don't know if that was said because our own regulatory people did not want to take on the responsibility of doing the technical work. But there is this to be considered: There will not be that many of these products coming on-line that we could go ahead and set up a research organization with an appropriate expertise and appropriate equipment for what may be a sporadic enterprise. But that is my only comment. We made the decision that we would take it to a group whose scientific credentials were impeccable, and we did not tell them that we wanted them to prove this virus is safe. We put the question in the honest way, "Would you have a look at this and tell us what it might do in a variety of animals?"

COLLINS: I think this is a possible alternative. All I would say is that industry, which is not known to be terribly altruistic, clearly feels that it is worth their while to keep a testing program like this available, even if it is only for occasional use. And I would say that if they feel it is worthwhile, given their cost-balance ratios, it might be worthwhile, whatever the inefficiency would be, for a regulatory agency to have a similar kind of program. But I think an alternative possibility would be, if they are adequately equipped, to have outside agencies do this. All I am saying is, and this is not to impugn the honesty and integrity of industrial research labs, the fact is that there exists the considerable possibility of conflict of interest, and from the safety standpoint, this is what we are all worried about. It seems that we ought to bend over backwards for the sake of care, particularly if we are worried about what the public is going to say relative to any possible problems that will arise in the future.

HEIMPEL: How long does this go on? There is no evidence that these viruses are harmful. They do not affect other animals. How long do we continue attempts to prove that they do?

COLLINS: I am not asking for absolute guarantees. All I am saying is it seems worthwhile to have confirmed the data that are available and I think that the point Dr. Ignoffo has brought up should address itself more to the second point that Dr. Joklik mentioned, namely what additional tests, now that technology is advanced further, should be used. I agree, I think additional tests are needed.

HEIMPEL: I agree too.

SUMMERS: I think what you are really asking is, can we reaffirm some of the critical safety experiments using more sensitive and appropriate techniques?

COLLINS: Yes, I think a lot of these techniques have the advantage of representing a form of basic research that is going to occur in a lot of laboratories, given adequate funding, not merely as a safety program, but as a part of basic research. I think from that standpoint, they are particularly valuable. They will probably be confirmed in a variety of labs and by a variety of workers using different cell systems and different viruses. All I am saying is whatever the criteria are going to be that are used in safety testing with new agents coming on, the results should be considered, and criteria confirmed.

SUMMERS: Then do we go back to Meinke's lead-off statement that our data appear adequate, but we need more research in the area of the biology of these viruses? What we need to do is identify that research in terms of immediate and longer-term goals.

FIELDS: I think we have answered the first part, in terms of immediate high priority items, in terms of reassociation kinetics and radioimmunoassays, and have discussed those back and forth. It might be reasonable to start making a list in terms of explicit recommendations for the next level of priority work. We are essentially saying that what has been done to date has been well done and is solid, and we are now just trying to set the next two levels of priorities. The immediate methodology that you have just discussed falls into the three groups of the hybridization methodology for DNA, RNA, and radioimmunoassays or other immunologic tests for proteins. Should we now identify a list of topics for more long-term development?

JOKLIK: I think it would be more profitable to appoint some subcommittees.

HOLOWCZAK: It is not clear to me what and how good immunological tests have been used and how good antisera have been for identifying these viruses.

IGNOFFO: This has been done but the tests have not been done with sensitive, specific antisera.

HOLOWCZAK: I consider it a very high priority to have agent-specific antisera available to identify these viruses and to monitor their occurrence in nature. This should be a part of any new safety testing procedures.

PART VII
PANEL DISCUSSION

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Discussion of Preliminary Draft of Panel Recommendations

Presenter: Robert E. Shope, M.D.

Baculoviruses have been shown to provide a promising alternative approach to pest control. Current evaluation methods suggest viruses are effective; furthermore, available data have not revealed any deleterious effects for other components of the ecosystem (i.e., other invertebrates, plants, and vertebrates, including man). Safety testing criteria should continually respond to improved technology. We draw attention to EPA guidelines for safety testing of baculoviruses, Section B-1, b-2. "It is important that the most sensitive methods for detecting virus replication are incorporated into the EPA guidelines for safety testing of baculoviruses." Recent developments in molecular biology have provided more sensitive and refined tools and offer the potential for testing at improved levels of specificity. By their implementation, the opportunity can be taken to improve further the safety tests for baculovirus pesticides.

We draw attention to the identification criteria listed on page 179 of Baculoviruses for Insect Pest Control. The recent development of restriction fragment analysis of a variety of DNA-containing viruses has provided a powerful new tool which should be included among the identification techniques. A fundamental requisite for safety is to demonstrate the lack of persistence or expression of the viral genome or parts of the viral genome in nontarget systems. Such tests should include two human cell types, as well as rodent, avian, and fish cells.

The development of such tests should embrace a variety of techniques such as molecular hybridization and radioimmunoassay. We strongly advise that EPA move with all speed to implement the above recommendations for safety involving identification, baculovirus genome tests, and sensitive methods for virus replication, at least by January 1979.

Research Program

To provide a continuing basis for improvement and an evaluation of new products, it is imperative that certain areas bearing on the safety of insect viruses should be more fully understood. A better understanding is needed of the replication and pathogenesis of baculoviruses in their natural hosts and cell culture systems. Also, the genetics of insect viruses and the development of specific genetic markers are vital for precise ecological study. For example, the possible range of interaction between the viral pesticide and other viruses in the biologic environment needs to be explored.

These general recommendations of the invited panel are intended to focus attention on areas urgently in need of research. They represent certain elements only of major issues raised in the panel discussion and are put forward as the basis for the formulation of a specific program at an early date. We feel that this could best be achieved by a permanent and independent advisory panel.

DISCUSSION

COLLINS: The two things I would like to open for discussion would be about what changes in safety testing procedures ought to be made. The only things being tested right now are the formulation and the active ingredient that goes into formulation.

The third possibility was that the most biologically pure preparation of the active ingredient also could be run through the same types of toxicity tests that are being done. That is one point that I think would be worth mentioning specifically. Second, I think it is very important to establish confirmatory testing of data.

HARRAP: I think as far as this is concerned we looked at the recommendations from the previous EPA meeting in Bethesda and found that several of the points you raised were covered in the recommendations there. That is why we draw attention to "Guidance for Safety Testing," Baculoviruses for Insect Pest Control, and cite certain paragraphs because we were not taken notice of the last time, and we hope that by repeating this, some note will be taken this time.

COLLINS: Except if you are drawing attention to a whole body of information, it is obviously unclear which aspects you think are important and which ones you don't. You might want to say that we are drawing attention to certain specific things, but if you are drawing attention to a whole body of information, the ones you want to stress are going to be lost.

IGNOFFO: Could we review each specific point in the recommendations?

SHOPE: It is stated in the first paragraph that, "Baculoviruses have been shown to provide a promising alternative approach to pest control. Current evaluation methods suggest viruses to be effective; furthermore, available data have not revealed any deleterious effects for other components of the ecosystem (i.e., other invertebrates, plants, and vertebrates, including man). Safety testing criteria should continually respond to improved technology. We draw attention to EPA guidelines for safety testing of baculoviruses, Section B-1, b-2."

IGNOFFO: Would you please read the section about that particular recommendation?

SHOPE: Section B-1, b-2: It's under "Data To Be Collected from Experimental Animals." "Attempts to isolate and detect insect virus inclusion bodies or virions in gastrointestinal tract, blood, urine, and selected body tissues which would normally be affected by a viral infection (e.g., lymph nodes, kidneys, cerebrospinal system, spleen, and liver) use the most specific and sensitive assay for virus detection." Attention should be focused on, "It is important that the most sensitive methods for detecting virus replication are incorporated into EPA guidelines for safety testing of baculoviruses."

PARTICIPANT: I don't really understand who is going to be responsible for the updating of these tests. It is certainly not going to be the licensee. I do not understand what organization or group is going to be responsible.

SHOPE: First of all, these are recommendations -- considerations that have been derived from our discussions for the past two days, which reflect what we feel -- the need for applying new and approved detection technology to evaluate safety on a continuing basis. These recommendations do not bring with them the immediate application of this technology. We have recommended that it now be superimposed upon future testing procedures or schemes.

COLLINS: If I understand it, the section you are drawing attention to really deals more with detection procedures than with what materials are going to be administered in these tests. Is that right?

SHOPE: That is correct.

COLLINS: This might be a possible place to bring in the testing of the...

IGNOFFO: That section, as he read it, specified the most active material.

COLLINS: No, not as I heard it. The most sensitive techniques were stressed.

IGNOFFO: I thought it also stressed that you utilize the most active substance -- the most biologically active fraction.

SHOPE: No, I do not believe that is right. Let me read it again from the "Safety Testing Procedure," concerning the data to be collected from the experimental animals. "The attempts to detect and isolate insect virus inclusion bodies or virions in gastrointestinal tract, blood, urine, and selected body tissues which would normally be affected by viral infection, use the most specific and sensitive assay for virus detection."

SMITH: If I might just draw attention to that -- Section A is where the material that is administered is described, and there might be the place to make a change. The very first sentence in Section A is titled, "Schedule of Feeding and Sacrifice," and it follows: "The insect virus is administered in a single dose to young adult laboratory animals, in both sexes." One could simply amend that by saying the most active insect virus, or the most active preparation, or the cleanest preparation, or however one might propose to do it, would be done in that fashion.

IGNOFFO: The licensee would also have to test the formulation to meet EPA requirements. If a person is trying to establish whether these agents are safe, he would not only want to meet the formulation requirements of EPA, but also to test the most infectious available entity.

COLLINS: I think one of the things we are trying to establish is what the intrinsic biological properties of these agents are, and although, clearly, testing the formulation is very good, as far as it goes, one of the things we are stressing here is that the biological systems are still relatively undefined, and I think this is very important information.

ENGLER: I would like to comment briefly on the issue of purity criteria of the inoculum which is used for testing. I think basically you should not be too concerned about this aspect and give the regulatory agency some latitude. In extreme cases, where the active ingredient which comes off the production line is 30 percent virus or even less, EPA has the foresight to ask for additional tests on the purified material. On the other hand, if that material that is produced is 95 percent virus and 5 percent insect fragments, almost anyone would be satisfied that that is as close as we can come to purified material. In reality, the test protocols take such variations into consideration. Making purity requirements too stringent may in fact box us in, and we may lose the ability of good scientific judgment for determining on what type of virus preparation the tests should be run.

COLLINS: Let me stress that I do not think we are looking for 100 percent purity, the important word in what I have been saying is "available," the most highly purified preparation available, recognizing all the problems inherent in finding this. The only thing I can say from what your comments have been is that, based on what I've heard in the last two days, EPA does not appear to have demonstrated the foresight in the past to really say that a virus preparation which is going into the formulation is not pure enough and that they are asking for something more. I see no evidence of that kind of control.

SHOPE: Can I ask Dr. Collins for a point of clarification? It seems to me, from what we know about what happens in the pathogenesis in the insect, that when a preparation is sprayed, it is consumed by the insect, and then at some point actual free virions appear in the hemolymph of the insect, or in tissues of the insect. Therefore, in order to test this properly in a tissue culture system, one would have to have free virions. The material that is being safety tested has, under the current procedures, a very small percentage of free rods and, therefore, if one does a test in vitro for integration of DNA into the genome, the basic starting material would have been polyhedra and thus inappropriate to initiate in vitro infection. Does this answer your question?

COLLINS: No. I am specifically referring to the animal toxicity testing that is being done already. If anyone wants to modify the tests that are being used, we can talk about that. That is not what I am talking about at the moment. All I am saying is that whatever criteria are being used right now for toxicity testing in animals, the four acute, the subacute, or whatever, the same series of tests should be run parallel with the best purified preparation available at that time. That's all I am saying, I am saying nothing about *in vitro* testing on tissue culture or anything like that.

IGNOFFO: Do you really want to formalize this into a regulation? Why not permit EPA the flexibility of deciding the best alternatives?

COLLINS: I have not seen where it has been done.

IGNOFFO: Oh, yes, it has been.

COLLINS: The preparations that have been spoken about so far in these last two days came across as being very...

IGNOFFO: Tests have been done with purified inclusion bodies, liberated virus particles, insect hemolymphs, etc. Not every test that was done included all these substances.

COLLINS: So if it has been done, I do not think there is any problem in including it in the guidelines that already exist. Let's formalize it then.

IGNOFFO: From a technical standpoint, purity is only a secondary factor to infectivity. I can give you a preparation that is 99.9% pure but is inactive.

COLLINS: The other word that was in the phrase that I have used is "biologically active."

ENGLER: Let me illustrate the problem with an actual case, the forthcoming registration of the gypsy moth NPV. The safety tests were done with a highly purified material. The registrant later decided that a less purified material will eventually be used. This has led to the condition where the safety tests were actually performed with a more purified material than that which is going to be used. The question then came up, "Must all the tests be repeated with the less purified material?" EPA decided that some tests had to be repeated and some did not; it became a matter of judgment. This is all I am asking, that a margin of judgment be maintained in conducting these tests.

COLLINS: I am not trying to suggest hard and fast rules. I think what I am suggesting fits in very well with what is being done and gives you as much flexibility as you want within the guidelines that you already have. I would ask and imagine that the preparation that you are talking about at the time it was submitted to you for testing was presumably the preparation that was going to be used in the formulation, and that is why if it was pure, it was because they were going to use a pure preparation in the formulation. If they had not, you never would have thought to look at that kind of preparation.

IGNOFFO: I would like to comment on the type of the inoculum which is used. I believe that virologists or registrants interested in evaluating risk factors do more than is required by regulatory agencies and will, in fact, test the most infectious agent as well as formulations. These materials, for reasons of time and cost, will not be tested in every animal system. Investigators will probably test only in the most critical system(s) in order to increase the probability of infection in a nonhomologous host.

COLLINS: There is no problem with that because the current guidelines would dictate that they would have to test the material that is going into the formulation. So, if they test 100 percent purified material, and later decide to use something that is 30 percent purified, then, as I understand the present guidelines, they have to go back and test that 30 percent because that is what is going into the formulation. I am suggesting that the testing of the most highly purified biologically active material available should be included in the toxicity testing along with the formulation and the material that is going into the formulation.

IGNOFFO: The recommendation is acceptable to me from a technical standpoint. I am not going to argue in EPA's behalf. In the final analysis it is their decision. But from a technical standpoint, I think your suggestion is desirable and has been used in the past.

SMITH: I think in terms of the administrator, we can recommend whatever we want. However, I think that it is important that we recognize that what I would consider a law is that whatever is put on the field is what has to be tested and that has to be given the most attention.

On the other hand, I think if there is any dangerous procedure, it would be to test the biologically purified material and then spray unpurified matter on the field. Parallel testing is extremely acceptable as far as I can see, but I think the real requirement must be that whatever is put on the field is what is tested for sure. And this precedent of having tested a purified product and then spraying an impure product is unacceptable.

SUMMERS: Shall this be included then as a recommendation?

RAPP: First of all, the group putting this together was conscious of the fact that EPA's main function concerns safety. And I think we should make the point very strongly that whatever we are writing here is strictly advisory. The third area that I would like to emphasize is that we were very careful not to get too specific. If you are going to start making highly specific, direct recommendations, you need much more information than I believe we have. You need the impact on the products, on costs, and on testing facilities. It does not simply involve putting in some more guidelines to run more tests. I think this is a very complex thing, and that is one of the reasons why we recommended a more permanent advisory panel to look into this to see if these guidelines should be written in great detail. To reamend all the previous documents that have been put together by various groups would, although we could have done that, have been an extraordinary mission.

I for one am very reluctant to put my name on highly specific recommendations. Most of these are general and trying to upgrade, in a general way, the level of safety testing to take into account newer technology and to urge new research programs that would enable better safety testing in the future as new products become available. I sympathize with Dr. Collins -- if we really start writing very specific recommendations into this, it would change the whole character of the document, the whole thing would have to be reworked and reexamined.

COLLINS: I view this as a fairly general recommendation. I am not saying anything about how the purified material should be prepared, what percentage of purity is necessary. I am putting it forward as a fairly general recommendation and I think, as I read the old recommendations that were written, the material that is being inoculated is being presented in a fairly general way. And I think it is in with that kind of terminology.

So, while I agree that this is not the appropriate forum for recommending very specific measures, I am really looking at this as a fairly general modification of what has already been done, without any specificity. This is where the flexibility comes in as to what is the most highly purified biologically active material available -- occluded virus and nonoccluded virus, or whatever. That is something for whoever is going to get involved with this to work out, and that is where flexibility comes in. So I say, if people feel that this is over-specific, they should vote against it. I am presenting it as a general modification and one that I think is important and has a reasonable amount of flexibility in it.

SUMMERS: Dr. Engler, do you visualize this as a general statement?

ENGLER: The more I hear Dr. Collins talk, the more I feel it is a general suggestion. The only reason why I was outspoken against restrictive and too specific purity criteria is that such criteria have a tendency to go beyond the scientific intent and will become legalistic. Once this has happened, the intended scientific flexibility and judgment are lost.

To summarize what we have done in the past and what will be done in the future: not necessarily all tests are performed with all different grades of purities. We are using bridging type tests, that is, some tests are done with more dirty material, some are done with more pure material, and some with both, giving us a basis for comparison. If there are significant differences, we will ask for more tests on the two components. If there are no differences, we can draw the conclusion that the state of purity does not affect the overall safety evaluation of the virus product. This is essentially what is being done and I hope that this is satisfactory.

COLLINS: The other point is that we should look at this meeting as something of a continuum in a series of meetings. I would like to see us moving in the right direction as far as safety testing goes. If a year or two from now, people refer to this meeting and the recommendations that were made, I hope they will be moving in the direction of more sensible testing. I think this discussion may be a small step in that direction, not a very great one, but a small one.

IGNOFFO: I think a lot of people, including me, would disagree with your comments on the validity and technical worth of the protocols of previous evaluations.

COLLINS: That is a separate question. That is not what I am dealing with. If you want to challenge the toxicity testing on the basis of whether it is scientifically valid or not, that is fine.

IGNOFFO: I was not challenging the tests or the protocols, but your introductory statements concerning the validity of these tests.

COLLINS: I am saying the material that is being used in the test should be expanded.

SUMMERS: You are right, Dr. Collins, this is a continuation of many things and I am sure it is going to continue. For example, the National Research Council of The National Academy of Sciences is putting together a study team to look at the same things that we are concerned with here, and perhaps in a more comprehensive way with more time available than we have had. I would have a tendency to support Dr. Rapp's comments. We spend a lot of time wanting to present a general statement that really focuses upon the very thing you are talking about, but doing it in a way that is compatible with the sequence of things -- the time that is available -- and yet, what we consider to be the urgent applications needed for improving safety or a more comprehensive evaluation of safety, not only now, but in the future. I myself would prefer to stick with the general statement, however, if this needs to be voted upon should we do it now?

COLLINS: I move that currently used animal toxicity tests be carried out with the most highly purified biologically active material available in addition to the tests that are already being done with the formulated material and the active ingredient used in the formulation.

CUNNINGHAM: Could I have some clarification on defining the term formulation, because I think everybody involved in the applied side of things knows that there is no ideal formulation yet available. We are all fiddling around with different things, and, in all probability, the formulations that are used this year will be altered by next year. Most of the things that are used in formulations at the moment are pretty innocuous materials, such as molasses, for example. Do I understand that a virus should be safety tested in the formulated state, and if in another two years there is a good change in formulation (for instance, one is using carbon again, another innocuous substance) that the whole routine should be run through again with the latest formulation? Or can the materials that are used in the formulation be safety tested independently from the virus? And then we can make up various permutations and combinations? How do we stand on that line?

ENGLER: That depends very much on what components are being used or substituted in a formulation. Certainly, if the formulation's "inerts" are as innocuous as molasses or milk sugar, a conclusion can be reached by the EPA as to how safe or how irritating these formulations are, whether or not additional tests should be run. If we cannot reach that conclusion, some or all tests pertaining to the formulation may have to be repeated. So it is an open question and an open-ended issue.

HUANG: What is meant by the "most purified" or "biologically active material"? Now as you all know, you can make up 2-3 micrograms of material that is highly purified and that is available. If you want about 30 grams perhaps, which is needed to run a toxicity test, that is a task of a different order of magnitude. So what kind of availability are you talking about?

COLLINS: I think this is the flexibility that we talked about. Whoever is going to be regulating this testing is going to have to decide that. I am trying to keep this as a general recommendation with flexibility for EPA, if that is who it is going to be, to decide how much they need and how pure it has to be. This may differ from virus to virus, and I would not even try to give you a number.

HUANG: So in other words, you are leaving it to the discretion and judgment of...

COLLINS: Exactly the same people whose judgment and discretion are now controlling it.

PULLIAM: You all ought to fill out your 171 Forms, send them to EPA, and come to work with us because I think you are begging a legalistic issue that is probably not appropriate for this particular meeting. You are, as many of you pointed out, making recommendations to EPA for safety testing and improvement on present methods that are being used to evaluate the safety of products that have been submitted for registration. I think that should be kept in focus. We are not making laws here and we are not making regulations -- we are not formulating guidelines that are going to be published in the Federal Register in the next couple of months. I think EPA welcomes any recommendations that you folks have to make, and I think they should be taken in that spirit. I do not think we need to argue the fine points of whether these things are available and how much they cost at this time. I think as you pointed out earlier, that is something that has to be done with more available time. I think the recommendations that you make are important, that they should be made, and that they should be transmitted so that they can be considered in perhaps a more practical light if necessary.

HARRAP: I think this whole conversation illustrates and, indeed, emphasizes very well what some of us were afraid of if we tried to get too specific. If you start to use a word like "purity" -- and the virologist in me has great attraction to that word purity -- as soon as you use it you will have to start defining it. You have to say what you mean, and then things start to change. So we have to have some guidance proposals to respond to that. For example, we have to decide whether, in toxicity testing, purity is important, and for virus replication tests whether it is important or not. So I really think this is what we are seeing, a good illustration of the value of trying to keep specific items out of these recommendations at this stage.

SUMMERS: All those in favor of Dr. Collins' proposed modification? Three. Those opposed? Thirteen.

SHOPE:

> "Recent developments in molecular biology have provided more sensitive and refined tools and offer the potential for testing at improved levels of specificity. By their implementation, the opportunity can be taken to improve further the safety tests for baculovirus pesticides. We draw attention to the identification criteria listed on page 179 of Baculovirus for Insect Pest Control. The recent development in restriction fragment analysis of a variety of DNA-containing viruses has provided a powerful tool that should be included among the identification techniques."

SHOPE: This is on page 179 under the heading of "Guidance for Safety Testing of Baculoviruses." I will read the paragraph: "The virus must be an insect pathogen, which is identified taxonomically according to host spectrum and serological and biochemical and biophysical tests." We want to call your attention to this new test which we feel should be included.

SHOPE: Next section:

> "A fundamental requisite for safety is to assure the lack of persistence or expression of the viral genome or parts of viral genome in nontarget systems. Such tests should include two human cell types, and rodent, avian, and fish cells. The development of such tests should embrace a variety of techniques, such a molecular hybridization and radioimmunoassay."

KNUDSON: I would just like a clarification of the first part of that sentence, for example, "A fundamental requisite of safety is to assure a lack of persistence." Does that imply that if there is persistence or an expression of a viral genome or any part thereof, then the agent in any cell line that you are using, nontarget systems, is not safe? Is that what that means?

SMITH: I think that if at this point we can identify a single criterion for potential problems, persistence has to be it. I do not know that that indeed will be the case, that if there is persistence in the form of an integrated genome and so on, it is unsafe. On the other hand, if there is a single criterion that I think can be identified at this meeting as a potential problem for further safety testing, I would say that persistence probably is as good a criterion as we can identify at this time. I think that that is what this recommendation is really saying.

KNUDSON: You redefined the word persistence in that discussion. If you want to define what you are talking about when you say persistence or expression, fine. I do not think it is very clear.

MEINKE: I think the statement is much too strong. You might have persistence of these DNAs, but for how long? Say it lasts for a week -- you can call it persistence, but it might clear. It could last 24 hours, whereas it could last for five. How long is persistence? Taking a ridiculous thing, suppose we grind up some cabbage leaves and extract the DNA. And then suppose I put that DNA into a million cells and it persists -- that would mean we should not eat cabbage anymore. It is much too strong a statement. It could stop the whole program, I think.

IGNOFFO: If you really want to make a distinction between the justification for the tests and actually doing the tests, I think we all agree that what we want is to do the tests. Thus, I suggest deleting the philosophical and justification phrases and stressing what the evaluation should include.

SMITH: No, again may I use the previous arguments all over again. We are trying to write in this communication recommendations to be taken to EPA for consideration. And I think the way it is written, the concerns of the advisory panel are underlined by this kind of phrasing. This is not going to be law, this is not going to be in the guidelines, as I understand it, at least that is what was said just a moment ago. And this is the way we express our concerns about this kind of biochemical or biological event, and nothing more than that.

IGNOFFO: Dr. Smith, what you have to ask yourself is, can you visualize a situation, any situation, where persistence would occur that would in fact completely prevent the development of this particular virus?

SMITH: Oh, yes indeed.

IGNOFFO: And, can you visualize a situation where persistence could occur, and yet not be a safety hazard?

SMITH: Yes, indeed.

IGNOFFO: If you can visualize these situations, then what we must not do is formulate a statement that would be all-inclusive and could in fact stop a program when persistence was demonstrated; yet this persistence did not present a safety hazard.

SMITH: That is right, but it is not the job of this panel to conduct those tests. What we are saying is, if persistence is demonstrated it is of concern, period.

KNUDSON: I think the point that I would like to make, the one word in the sentence that I think throws the whole context off is the word "lack." If you could change that maybe to something like "to examine the extent of any possible" or "what is the significance thereof." The word "lack" is the problem.

IGNOFFO: I think it makes it a hard and fast rule that does not leave the possibility of backing out because we do have persistence and yet there is no safety hazard.

HARRAP: May I explain the reason for this statement? Dr. Joklik was virtually adamant that a specific recommendation like this was made. We had some discussion about its implications. He felt maybe it would not totally rule out a specific virus in a certain instance, but he wanted this recommendation in so that if there was any persistence or expression of baculovirus genome in nontarget cells, a more thorough look would be taken at the situation by having the recommendation there.

IGNOFFO: That is exactly what that will do! If we are primarily concerned with evaluating the existence of persistence, then O.K. If tests are recommended that will evaluate persistence, then at least the next step (if persistence does exist) is to decide whether to proceed. But if it is included as recommended, I think there is only one recourse after an evaluation has been made -- to stop everything.

HARRAP: No, that was not his feeling. What he wanted to do was to stop things while people took a more thorough look at them.

MEINKE: I think it is too strong. Can't we just say something to the effect that "a fundamental concern of"?

COLLINS: Something like, "The fundamental concerns of safety require the investigation of the question of persistence..."

SHOPE: Full quote:

> "A fundamental concern for safety requires the investigation of the question of persistence or expression of the viral genome, or parts of the viral genome in nontarget systems. Such tests should include two human cell types, and rodent, avian, and fish cells. The development of such tests should include or embrace a variety of techniques such as molecular hybridization and radioimmunoassay."

MEINKE: Now I think the second part of that is also too strong and too specific. I do not think we should spell out two of these kinds of cells, one of this and that. I think we should just use nontarget systems and leave it up to EPA to decide what are the nontarget systems. Can't we just stop it at nontarget systems?

FIELDS: Well, as a minimum you want a fibroblast and a epithelial cell and this is not an absolute number, but certainly I believe that all of us felt that this was a minimum screen that certainly could and probably should be supplemented. But this was a relatively specific and strong feeling that most of us had, that this really was in many ways the crux of the problem of altered host range and if there was to be any major, however remote, issue of safety, this would be the most crucial but specific recommendation to come out.

IGNOFFO: You are concerned with cell type?

FIELDS: Yes, well...and I think it was really the feeling that if one were to choose there would be absolutely no way of having a single cell type that could cover it. Two is really a minimum, but at least one could cover fibroblast and epithelial. But it would be advisable in developing this to broaden it and add further cells. But I think to leave this too general would be to dilute what really is the remote but focal issue that we are coming to of tests that can be added, and really further refine safety.

HARRAP: In fact, in my notes I have the words "at least." I do not know whether it was noted, but we did say it.

MEINKE: I would like to point out that in almost every mammalian system I can think of, you can use a monkey cell line to mimic any kind of occurrences in other cells. I am not saying that there are not exceptions, but whatever happens in human cells in cultures, usually happens in monkey cells in cultures. So, I do not know why you would really have to lock in on human cells for one thing. I am trying to think of a system where one virus does not replicate on the monkey cell line and does replicate on a human cell line. Maybe I should say primate cell lines. I hate to see anything just locked in like that where you just have to do those experiments like that. It seems, it is such a firm statement, that's all.

RAPP: I will give him a very famous example. Herpes simplex virus probably grows in more types of cells than any other mammalian virus. It does not grow in rhesus monkey cells, which was a favorite cell for most investigations until about fifteen years ago. So, examples exist, and I could give you others, but that is the most obvious one.

MEINKE: Yes, but there are a lot of examples in reverse where these viruses do grow better in other primates besides human cells.

RAPP: In the final analysis, the species we want to protect most is the human species and that is the reason for using human cells.

IGNOFFO: If that is the ultimate concern, why not concentrate on primate lines? Forget about avian, mammalian, and fish lines. Let's assume that we do not get viral replication or cytopathic effect in primate cell lines but do get effects in other vertebrate lines. Then what?

SMITH: Well, again, getting back to the spirit of what we are trying to prepare here, let's propose now for a moment this particular virus integrates and persists in fish cells, but does not in chicken or mammalian or human cells. Then you put it to the EPA and they make the judgment that it is not important in this case. On the other hand, if you present data that says the genome integrates into mammalian, into human cells, as well as fish and insect cells, then it is a different ball of wax. And what we are saying in this communication is that this information should be available for safety testing.

RAPP: Well, what we had hoped, without listing 500 cell types, was that the EPA would use its latitude. If, for example, you were spraying an area in which dairy cattle grazed regularly, one might use bovine cells. We were looking from the point of view of various other economically important and ecosystem important species. This was just a suggestion that is a minimum and the reason is that these types of species are found in areas that would generally be sprayed. I mean, there would be water in most of them, and so on. It is rather arbitrary to say fish because we knew that there were fish cell lines available. But if I were spraying in an area where I knew there were a lot of cattle, I would try to use some bovine cells.

SHOPE:

> "We strongly advise that EPA move with all speed to implement the above recommendations for safety involving identification, baculovirus genome tests, and sensitive methods for virus replication, at least by January 1979."

COLLINS: I hesitate to bring this up, but I really think we should address the question of confirmatory testing, whether it be by EPA or by anybody else. I think this is extremely important and to emphasize what was said before, we are making recommendations and not using the force of law. The point is that if we do not make these recommendations, presumably they will not even be discussed by EPA since they are not even going to come before them. I think this is a very, very important concern, and I think it is one that should be brought to EPA's attention. I recognize that implicit in this would be increased funding, increased staffing, but again we are here to make recommendations on what would be the most rational approaches to take from this point on, and I think that this is an extremely important one.

ULVEDAL: I have been pretty quiet in this discussion because I am on the research side of the house and not the regulatory side of the house to which most of these questions have been addressed. However, from the research side, I would like to hear as many specific recommendations and strong recommendations as possible. That gives us the option to examine, evaluate, and then sift out what we can do and what we cannot do. So I would like to hear as many specific recommendations as possible.

SUMMERS: Would it be possible for you to modify that statement to include something like "most critical" tests?

COLLINS: I would say before an agent is registered for use as a viral pesticide that data relevant to the most critical safety tests should be confirmed either by a regulatory agency or outside independent investigators. EPA does not have to feel that certain of the data are inconclusive. I mean, they can take data that look perfectly reasonable on the surface. The fact is that they have to decide which of those tests are most critical.

SUMMERS: "Before a biological agent is registered as a viral pesticide, the data most relevant to the safety test should be reconfirmed by EPA."

Is that a motion to be added to this section?

COLLINS: Yes, I move that the motion be added.

IGNOFFO: Dr. Engler, what are your comments on this? You have been very quiet. I would like to hear what you have to say.

ENGLER: I made my comments before and as long as it has been said around this table that these are recommendations which may or may not be implemented, I have no comments on that.

IGNOFFO: Just out of curiosity, would you recommend that the recommendation on reconfirmation of tests' results be implemented?

ENGLER: No.

SUMMERS (restatement of the motion): "Before a biological agent is registered as a viral pesticide, the data most relevant to the safety tests should be reconfirmed by EPA."

ANGUS: Mr. Chairman, could you entertain some advice from the floor?

SUMMERS: Yes.

ANGUS: Although I know it is not directly pertinent to your consideration today, there are some international implications in the wording of this because in some jurisdictions, and I am not aware of what is the rule here, it is the premise that the applicant is responsible for whatever costs arise from trying to find the registration for something. Now as I hear this wording, it means that EPA will have to go out and do the tests itself, and this implies that EPA is now going to set up its own testing facility, that they will have protocols and results and data submitted to them, and that they will decide to do some part of it or all of it all over again. Is that what is implied?

COLLINS: No, I think the discussion we just had made it very clear that that could either be done in-house or they could send that out.

SHOPE: I think I need some clarification here also. Let us assume there is a virus which is proposed for registration by the Forest Service and that presumably the cost for the development of that registration would be borne by public funds. You are then suggesting that we use more public funds to confirm what a government agency has already found to be true. I personally feel that that would be an unwarranted use of public funds.

COLLINS: I would consider that an appropriate use of public funds. I think that situation is not going to be the norm, or at least it will be a mixture of that and where the initial testing has been done with private money.

SUMMERS: Once again I think the recommendation has inherent in it that flexibility because it states that the data most relevant to the safety tests be reconfirmed. So I think that is left to EPA's, or whomever's, discretion. All in favor of this motion? Twelve. Those opposed? Two.

SHOPE: There was a question about January 1979.

> "We strongly advise that EPA move with all speed to implement the above recommendations for safety involving identification, baculovirus genome tests, and sensitive methods for virus replication, at least by January 1979."

RAPP: The fact is that there were many people on the panel who felt that we ought to put in January 1979 because the technology is so advanced. No one is asking for results by January 1979, just that things be under way, so to speak, and that some of these tests be put into operation. That is a very, very loose kind of recommendation which is simply intended to call attention to the fact that EPA ought to be heading in that general direction. That is all it really implies.

IGNOFFO: If I had a new virus, how long would it take me to develop the radioimmunoassay technique?

RAPP: Well, to some extent it would depend on how well it would grow in your test systems, how fast you could purify the proteins, but if you already have the technology, and apparently the technology that purifies the various proteins associated with baculoviruses is available, I do not think it would take long.

HARRAP: Developing the radioimmunoassay...four months.

SUMMERS: Four to six, because you would want time for standardization.

FALCON: I would like to know why it is necessary to specifically tell EPA to implement these things. Isn't it assumed that the conference, the fact that EPA gave sanction to this conference, aren't you begging the question? I see no need at all for that particular sentence.

SHOPE: At least a partial response, the document, which is an appendix to the book, Baculovirus Safety Testing, has not been implemented...at least parts of it. And I think that this is an effort at least to have specific areas implemented.

FALCON: The fact that it has not been implemented does not mean that EPA has not reviewed that information and considered it and taken from it what they feel they can utilize. I think in this case you are making recommendations and then you are moving one step further where you say, "You better implement these recommendations." Now that is going beyond what a recommendation is designed to do, I feel.

HARRAP: No, I do not agree with you. The historical fact is that we have made recommendations before. All that I have seen at this meeting, and what has happened since, is that EPA has taken no notice of it. There seems to be no rational attempt to identify the viruses, as far as I can see. So we are drawing attention to this again. This time we are pointing out certain techniques by which this can be done, which we think are practicable. If they choose to disregard it again, that is their affair, but I think it is our duty to recommend. We are recommending again what we recommended before.

ENGLER: Since the Bethesda meeting, EPA has actually registered only one virus, the tussock moth virus, so, implying that the guidance or recommendations of the Bethesda meeting have not been implemented is not quite fair; how can EPA implement recommendations if there are no viruses registered?

HARRAP: In that instance did you have certain criteria for identity? Did you have a battery of data on the virus properties and, for example, antisera against components of the virus before you agreed to register it?

ENGLER: One thing that the people assembled in this room have to realize is that we need almost a "cookbook" type recommendation for identification, as well as for any other tests such as those that are suggested by Dr. Meinke and colleagues. Before we standardize this approach, we cannot proceed, we cannot tell the registrant, whoever he may be, "Do something along the line of DNA identity." This is one thing that I have to stress here, and that all the people in research have to keep in mind, the tests which will be used eventually must be standardized and tested for their predicted value.

HARRAP: But can't you contract this work out to competent labs? The Ministry of Overseas Development in England contracts out characterization work to us and pays us for it, and then they contract out the toxicity work to MRE, in Porton Down, and pay them for it. They do not have all the expertise themselves.

SMITH: One of the things we are recommending for implementation by this date is a set-up of a sort of permanent advisory panel. I would envisage one of the functions of this permanent advisory panel is to make specific "cookbook," if you wish, recommendations to the EPA. What we are saying by setting this date is that this is a reasonable time by which this panel should be well under way. Other things that we think are more pressing, in terms of safety testing, etc., are also included in this timetable. So I think that within the confines of this short time that we have here, and this is a brief document, I do believe this is a reasonable timetable with which this permanent panel can be set up. You can ask that panel to do whatever you wish, but one of these things is what you have suggested.

SUMMERS: Is there a motion to remove that date or to modify that statement? Is there a specific motion before us on that statement at all? Is there additional discussion?

SHOPE:

> "Research Program. To provide a continuing basis for improvement and an evaluation of new products, it is imperative that certain areas bearing on the safety of insect viruses be more fully understood: 1. A better understanding is needed of the replication and pathogenesis of baculoviruses in their natural hosts and cell culture systems...."

GRANADOS: I suggest that we drop back to baculoviruses and insert insect viruses to make that more general.

SHOPE:

> "To provide a continuing basis for improvement and an evaluation of new products, it is imperative that certain areas bearing on the safety of insect viruses should be more fully understood. A better understanding is needed of the replication and pathogenesis of baculoviruses in their natural hosts and cell culture systems," and the suggested modification would be "A better understanding is needed of the replication and pathogenesis of insect viruses in their natural hosts and cell culture systems."

IGNOFFO: The primary concern at this stage is for those viruses we believe can be developed feasibly. Thus, I believe we should continue to stress the baculoviruses. I am fully in favor of a study of all insect viruses, their modes of replication, pathogenicity, the whole pattern of replication, and specificity. But I think this conference directly relates to the baculoviruses.

SMITH: Now, I would like to disagree with that. I think it is relevant to safety to know what insect viruses do, and one of the things that can happen is that you can spray a forest or a family with an uncharacterized virus that you really should know something about. How do you prevent its replication? Do you have antiserum?

IGNOFFO: How do you know something about an unknown virus?

SMITH: I did not say "unknown," I said "uncharacterized." Let's say, for example, we had the cricket paralysis virus unwittingly sprayed on the people. Are antisera available, how does it replicate, what does it replicate in? That kind of information is necessary. What you are trying to say here, I hope, is that the panel advises further research along lines that may be relevant to safety.

COLLINS: What about a compromise, something like, since the word "insect viruses" is used in either the previous sentence or the previous phrase in that sentence..."of these agents with particular emphasis on baculoviruses"?

SHOPE: I can read it if you like.

> "To provide a continuing basis for improvement and an evaluation of new products, it is imperative that certain areas bearing on the safety of insect viruses, with particular emphasis

on baculoviruses, should be more fully understood. A better understanding is needed of the replication and pathogenesis of viruses in their natural hosts and cell culture systems. The genetics of viruses and the development of specific genetic markers are vital for precise ecological study. For example, the possible range of interaction between the viral pesticide and other viruses in the biologic environment needs to be explored."

SUMMERS: All in favor? Unanimous. Proceed.

SHOPE:

"These general recommendations of the invited panel are intended to focus attention on areas urgently in need of research. They represent certain elements only of major issues raised in the panel discussion and are put forward as the basis for the formulation of a specific program at an early date. We feel that this could be best achieved by a permanent and independent advisory panel."

SMITH: I move that we unanimously adopt it.

SUMMERS: Is there a second to that? All in favor? It is unanimous.

I just want to say thanks to the Environmental Protection Agency for providing the opportunity for an entirely new forum or atmosphere in which to think intelligently and discuss the use of viruses as biological alternatives for chemical pesticides. I want to thank my colleagues who took part in the formal presentation and participations. We have been working together for some time. For those of you not on the panel who do not know us well, I want you to know that they really did take this task seriously, and they did a superb job.

I want to thank the members, many of whom are not here now, of the Virology Advisory Panel. As I said earlier, only two out of the original 12 declined. From your comments, my interpretation is that from the standpoint of or evaluation of basic research, you have been very complimentary, and I think you recognize what the important problems are in our programs. You really revealed through an honest response and evaluation the promise of this area of virology, and I think you perhaps recognize the promise and the problems of this area better than some of us do, perhaps even the government.

I am personally gratified for this exchange. It has been a very intellectual one, and I hope we have the opportunity to do it again. I hope we now can begin to consider you as colleagues, as I hope you will consider us colleagues and virologists.

To the EPA, I think it is clear that all has been said that can be said. The serious problem that we have is really implementation. We keep saying this at every program, every meeting, over and over again. And my final comment to the Environmental Protection Agency is that I would hope that you would somehow identify your responsibility in this area and provide a mechanism to deal with it.

CURLEY: Thank you, Max. I, too, would like to, on behalf of the Office of Research and Development and the Health Effects Lab at Research Triangle Park, North Carolina, thank you and your colleagues for putting this symposium together, and for the recommendations that will be forthcoming from you. I would like to point out here, and I register only one concern that I would like to share with you.

We are in the Office of Research and Development. Our primary responsibility is research, and Reto Engler's office is the office that is a program office, the Office of Pesticide Programs. We respond to two different administrators. We have not attempted to provide a forum for looking at basically the research in this area. You have not only done that, you have also looked at some of the regulatory aspects of the viral pesticides program. So we got two things, we got recommendations on the regulatory process, and we got recommendations on research. Through my administrator, we will provide the recommendations that you have made to us on the regulatory process to Reto's administrator, and hopefully, they will consider, or give due consideration to all of these recommendations that have come out of this symposium.

Again, I thank you and your colleagues and we hope that we will again be able to provide further symposia, further research through the Office of Research and Development, and hopefully, through OPP, through Reto Engler's group. Both his group and my group can respond to various aspects of these recommendations put to us. We will look forward to working with you again and I hope that Reto Engler and his group will also work with you.

ENGLER: From all the conferences on safety aspects of insect viruses held in the past, I feel that this has been the most rewarding one, primarily for the reasons which Dr. Curley mentioned. It has become clear to the scientists and to the virologists that there are two different needs related to the viral pesticides.

The recommendations clearly state that we should categorize the basic research and the testing for regulatory purposes that need to be done. The testing presently done was found largely acceptable, some additional testing was highly recommended to be included in the near future, and, in addition, basic research is recommended as a long-term goal. I also would like to reemphasize that any safety test should be standardized and tried out as completely as possible. This applies to identification as well as to the sophisticated virus-cell interaction tests.

Along with that, I would also make a statement regarding our plight in the registration part of the pesticide effort of EPA. One must consider that we are working very close to the legal side of pesticide regulation. If in the future this panel of experts recommends a test and EPA is going to adopt the test, it will be difficult to deviate from the requirement unless flexibility is built into it. That goes back to how many tissue cultures we are going to use and the other topics that were discussed around the table this morning. So please keep that in mind, that when a test becomes a requirement it is not only a scientific requirement, it is also a legal requirement. Along with these thoughts, maybe some of the terms need to be redefined, namely, guidelines. Guidelines have in the past become a legal document, whereas guidance is not considered a legal document.

As a final statement I would strongly support what is also contained in the recommendation, that this or a very similar panel of experts will continue as an advisory group to finalize those standard tests and to finalize the future research needs.

SUMMERS: I am assuming that this session is finished.

Conference Attendees

DR. GEORGE ALLEN
Professor
Department of Entomology and Nematology
University of Florida
3103 McCarty Hall
Gainesville, Florida 32611

DR. MARIO ANDRES
University of Toronto
540 College Street
Toronto, Ontario
CANADA

DR. THOMAS A. ANGUS
Insect Pathology Research Institute
Post Office Box 490
Sault Ste. Marie, Ontario, P6A 5M7
CANADA

DAVID J. BROWN, M.SC.
Plant Products Division
Canada Department of Agriculture
Ottawa, Ontario K1A 0C5
CANADA

DR. JOHN A. COUCH
Research Pathologist
U.S. Environmental Protection Agency
Environmental Research Laboratory
Gulf Breeze, Florida 32503

DR. JOHN C. CUNNINGHAM
Insect Pathology Research Institute
Post Office Box 490
Sault Ste. Marie, Ontario, P6A 5M7
CANADA

DR. S.C. CHOI
University of Toronto
540 College Street
Toronto, Ontario
CANADA

AUGUST CURLEY
Chief, Toxic Effects Branch
U.S. Environmental Protection Agency
Health Effects Research Laboratory
Environmental Toxicology Division (MD66)
Research Triangle Park, North Carolina 27711

DR. WALTER DOERFLER
Professor of Genetics
University of Koln
Weyertal 121
5000 Koln 41
WEST GERMANY

DR. PETER FAULKNER
Professor
Department of Microbiology
Queen's University
Kingston, Ontario, K7L 3N6
CANADA

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DR. JOHN J. HAMM
Research Entomologist
U.S. Department of Agriculture, ARS
Southern Grain Insects Research Laboratory
Tifton, Georgia 31794

DR. A.M. HEIMPEL
Chief, Insect Pathology Laboratory
Plant Protection Institute
U.S. Department of Agriculture, ARS
BARC West
Beltsville, Maryland 20705

DR. KENNETH J. HOOD
Office of Research and Development
U.S. Environmental Protection Agency (RD-683)
401 M Street, S.W.
Washington, D.C. 20460

DR. H.T. HUANG
Program Manager
National Science Foundation
1800 G Street, N.W.
Washington, D.C. 20550

DR. ROBERT L. KAISER
Director
Bureau of Tropical Diseases
Center for Disease Control
1600 Clifton Road, N.E.
Atlanta, Georgia 30333

DR. WILLIAM H.R. LANGRIDGE
Virologist
Boyce Thompson Institute for Plant Research
1086 North Broadway
Yonkers, New York 10701

DR. HORACE MAZZONE
Forest Insect and Disease Laboratory
U.S. Department of Agriculture
151 Sanford Street
Hamden, Connecticut 06514

DR. A.H. MCINTOSH
Waksman Institute of Microbiology
Rutgers University
New Brunswick, New Jersey 08903

DR. LOIS K. MILLER
Assistant Professor
Department of Biochemistry
The University of Idaho
Moscow, Idaho 83843

DR. H.G. MILTENBURGER
Professor
Institute of Zoology
Technical University
D-6100 Darmstadt
WEST GERMANY

DR. JOHN D. PASCHKE
Professor
Department of Entomology
Purdue University
West Lafayette, Indiana 47907

DR. ROGER L. PIERPONT
Lead Registration Support Section
Ecological Effects Branch
Criteria and Evaluation Division
U.S. Environmental Protection Agency
Crystal Mall, Building #2, Room 824
Arlington, Virginia 20460

DR. JEAN E. PULLIAM
Chief, Pesticide Research Coordination Staff
Office of Pesticide Programs
U.S. Environmental Protection Agency
East Tower 501 (WH-566)
401 M Street, S.W.
Washington, D.C. 20460

ANN L. RALSTON
Microbiologist
U.S. Environmental Protection Agency (MD67)
Research Triangle Park, North Carolina 27711

RUSSELL REGNERY
Department of Microbiology
Rutgers Medical School
College of Medicine and Dentistry of New Jersey, Box 101
Piscataway, New Jersey 08854

DR. DONALD W. ROBERTS
Insect Pathologist
Boyce Thompson Institute for Plant Research
1086 North Broadway
Yonkers, New York 10701

DR. K.R. ROZEE
Professor and Head Microbiology/Medicine
Dalhousie University
Sir Charles Tupper Building
Halifax, Nova Scotia, B3H 4H7
CANADA

DR. PATRICIA L. RUSSELL
Staff Biologist
Special Science Programs
American Institute of Biological Sciences
1401 Wilson Boulevard
Arlington, Virginia 22209

DR. ERNST-FRIEDRICH SCHULZE
Hoechst Aktiengesellschaft
Pflanzenschutzforschung Biologie
Postfach 800320
Frankfurt/Main 6230
WEST GERMANY

DR. MARTIN SHAPIRO
Research Entomologist
U.S. Department of Agriculture, ARS
Building 1398
Otis Air Force Base, Massachusetts 02542

DR. T.R. SHIEH
Manager
Biological Research and Development
Sandoz Incorporated
15995 SW 78th Place
Miami, Florida 33157

GALE E. SMITH
Department of Entomology
Texas A&M University
College Station, Texas 77843

DR. GORDON R. STAIRS
Professor of Entomology
Ohio State University
1735 Neil Avenue
Columbus, Ohio 43210

DR. D.B. STOLTZ
Assistant Professor of Microbiology
Dalhousie University
Sir Charles Tupper Building
Halifax, Nova Scotia, B3H 4H7
CANADA

DR. FRODE ULVEDAL
Acting Director
Health Effects Division
U.S. Environmental Protection Agency (RD 683)
401 M Street, S.W.
Washington, D.C. 20460

DR. STAN WEBB
Department of Microbiology
Medical College of Virginia
Box 487
Richmond, Virginia 23298

DR. H. ALAN WOOD
Associate Virologist
Biological Control Program
Boyce Thompson Institute for Plant Research
1086 North Broadway
Yonkers, New York 10701